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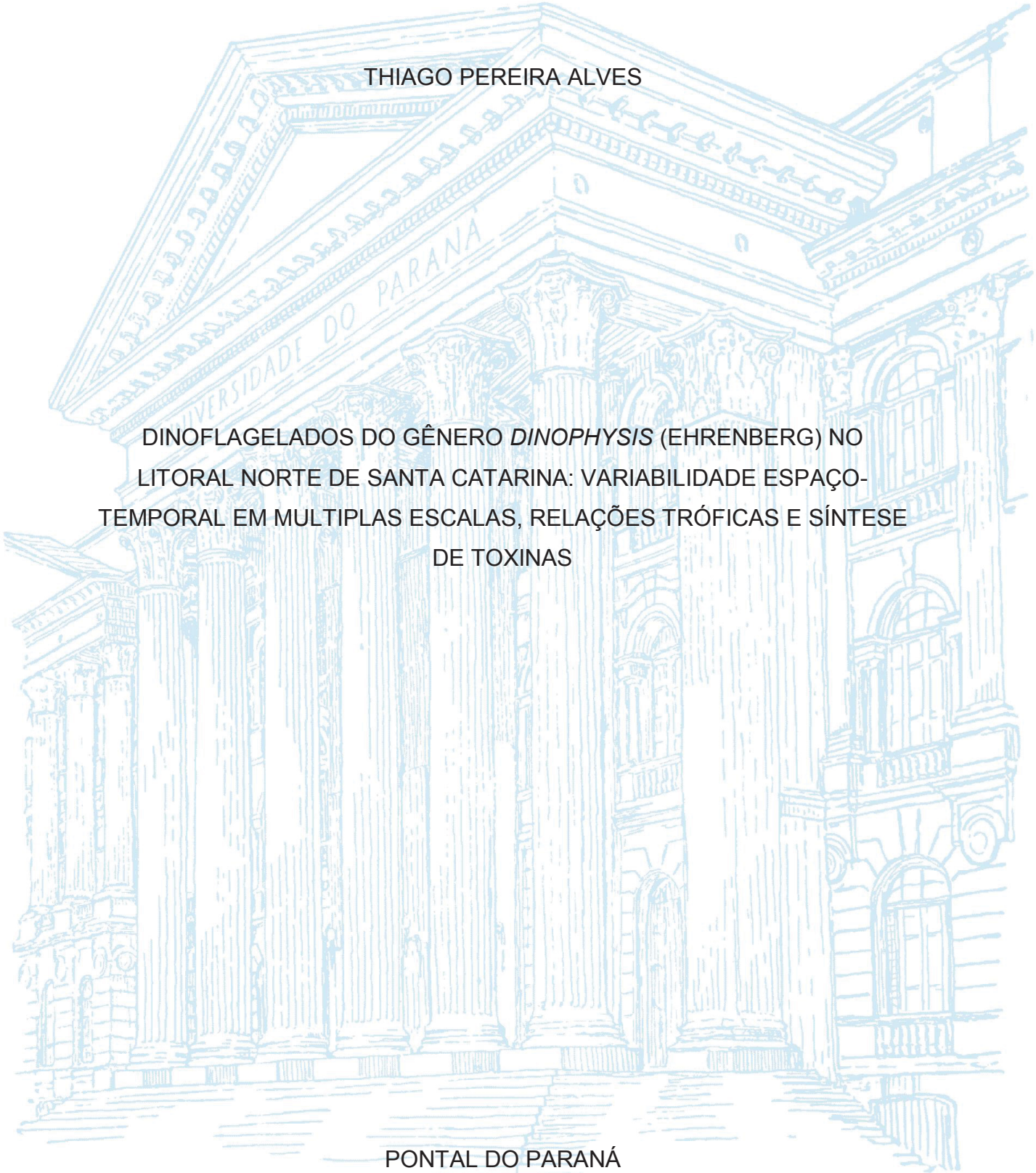
THIAGO PEREIRA ALVES



DINOFLAGELADOS DO GÊNERO *DINOPHYSIS* (EHRENBERG) NO LITORAL NORTE DE SANTA CATARINA: VARIABILIDADE ESPAÇO-TEMPORAL EM MULTIPLAS ESCALAS, RELAÇÕES TRÓFICAS E SÍNTESE DE TOXINAS

PONTAL DO PARANÁ

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Orientador: Prof. Dr. Luiz L. Mafra Jr.

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em SISTEMAS COSTEIROS E OCEÂNICOS da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **THIAGO PEREIRA ALVES** intitulada: **Dinoflagelados do gênero *Dinophysis* (Ehrengberg) no litoral norte de Santa Catarina: variabilidade espaço-temporal em múltiplas escalas, relações tróficas e síntese de toxinas**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
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LISTA FIGURA

LISTA TABELA

LISTA DE ABREVIAÇÕES

AO – Ácido Ocadaico

APP – Água da Pluma do Prata

CIDASC – Companhia Integrada de Defesa Agropecuária de Santa Catarina

DSP – *Diarroethic Shellfish Poisoning*

DTX – Dinofisistoxina ou *Dinophysistoxin*

ENSO – *El Niño Southern Oscillation*

EPAGRI – Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina S/A

FAN – Floração de Algas Nocivas

HAB – *Harmful Algae Bloom*

HPLC – *High Performance Liquid Chromatography*

IFSC – Instituto Federal de Santa Catarina

INMET – Instituto Nacional de Meteorologia

INPI – Instituto Nacional de Propriedade Industrial

LANF – Laboratório de Algas Nocivas e Ficotoxinas

LAQUA – Laboratório Oficial de Resíduos e Contaminantes em Recursos Pesqueiros

LC MS/MS – *Liquid Chromatography tandem with Mass Spectrometry*

MAPA – Ministério da Agricultura, Pecuária e Abastecimento

NSP – *Neurotoxic Shellfish Poisoning*

OA – *Okadaic Acid*

PCS – Plataforma Continental do Sul

PCSE – Plataforma Continetal do Sudeste

PNCMB – Programa Nacional de Controle Higiênico e Sanitário de Moluscos Bivalves

PPW – Plata Plume Water

PSP – *Paralythic Shellfish Poisoning*

PTX – Pectenotoxina ou *Pectenotoxin*

RENAQUA – Rede Nacional de Laboratórios da Pesca e Aquicultura

SPM – *Suspended Particulate Matter*

YTX – Iessotoxina ou *Yessotoxin*

ZCBM – Zona de Convergência Brasil-Malvinas

LISTA DE SÍMBOLOS

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RESUMO

Cultivos de moluscos bivalves representam uma importante atividade econômica no litoral de Santa Catarina, sul do Brasil. Florações de algas nocivas, especialmente do género *Dinophysis*, com consequente contaminação dos moluscos por toxinas causadoras da síndrome diarreica dos moluscos (DSP) são a principal causa de impactos econômicos negativos ao setor produtivo. Nesta Tese, foi investigada a distribuição espacial e temporal com diferentes escalas de abordagem observando aspectos ecológicos como a mixotrofia e a síntese de toxinas em *Dinophysis* spp. *D.* cf. *acuminata* foi a espécie de maior frequência e abundancia dentre as espécies do gênero, mas *D. caudata, D. tripos, D. scrobiculata, D sacculus* e *Phalacroma rotundatum* também foram observados. O período de mais ocorrência das espécies de *Dinophysis* ocorre entre o início do inverno e o início do verão. Florações de *Dinophysis* com a detecção de toxinas DSP, tem maior probabilidade de ocorrer à partir de julho, sendo 2007, 2011 e 20016 os anos que apresentaram os eventos de maior toxicidade. Somente o ácido ocadáico (AO) foi detectado acumulado na fauna marinha, tanto na forma livre como metabolizada (esteirificada). Os moluscos bivalves foram os organismos que acumularam esta toxina mesmo durante baixíssimas abundâncias de *Dinophysis* spp. Durante uma floração o AO pode acumular também em outros componentes da fauna marinha como cracas, poliquetas, anfipodes carangueijos, camarões e peixes. A co-occorrência de *Dinophysis* spp. com o ciliado *Mesodinium rubrum* evidencia a relação trófica presa-predador observado, principalmente, nas abordagens de menor escala. O fenômeno climático global do *El Niño* (ENSO), demonstrou ter associação com os eventos de floração quando apresenta uma condição de anomalia negativa (*La Niña*), embora algumas espécies de *Dinophysis* ocorram durante todo o ano.

Palavras-Chave: FAN, DSP, ácido ocadáico, mixotrofia, fauna marinha

ABSTRACT

Marine shellfish aquaculture represents an important economic activity in the State of Santa Catarina, Southern of Brazil. Harmful algal blooms (HABs), especially of *Dinophysis* genus, with consequent contaminate of bivalves by diarrheic shellfish poisoning (DSP) toxins are the main factor of negative economic impacts on the productive sector. The spatial and temporal distribution was investigated in different scales observing some ecological aspects of these dinoflagellates such as mixotrophy and toxins profile. *D.* cf. *acuminata* had the highest frequency and abundant among *Dinophysis* species, but *D. caudata, D. tripos, D. scrobiculata, D sacculus* and *Phalacroma rotundatum* were also observed*. Dinophysis blooms* occurs from early winter to early summer, and the bloom with higher severity were observed in 2007, 2011 and especially in 2016. Only the okadaic acid (OA) was detected in the marine fauna, both in the free form as it was metabolized (esterified). Bivalve molluscs accumulated OA even during very low abundances of *Dinophysis* spp. When the bloom occurs, the OA might be accumulating in several marine organisms, such as barnacles, amphipods, polychaeta, crabs, shrimps and fishes. Observed in the approaches of smaller scales, the co-occurrence of *Dinophysis* spp. with the ciliate *Mesodinium rubrum* shows evidence of the prey-predator trophic relation. The global climatic phenomenon of *El Niño* (ENSO) has been shown to be associated with *Dinophysis* blooms mainly at negative anomaly condition (*La Niña*), although *Dinophysis* occur throughout the year.

Key-words: HAB, DSP, Okadaic acid, Mixotrophy, Marine biota

SUMARIO

INTRODUÇÃO E HIPÓTESES

Microalgas pertencentes à classe Dinophyceae (dinoflagelados) compõem um grupo diversificado de organismos unicelulares, caracterizados pela presença de dois flagelos distintos – um transversal com dupla fileira de mastigonemas, e outro longitudinal – e de um conjunto de pigmentos fotossintéticos composto por clorofila-*a*, c_2, carotenoides, e xantofilas (Sartory, 1985; Balech, 1988; Faust & Gulledge, 2002). De acordo com o perfil de pigmentos fotossintéticos, os dinoflagelados são divididos em dois grupos, que descendem de ancestrais diferentes, sendo o tipo 1 dotado de plastídios contendo o carotenoide peridinina, pigmento exclusivo e associado a uma linhagem que descendeu de algas vermelhas, e o tipo 2 com plastídios contendo carotenoides e xantofilas derivados de haptofitas (Stiller & Hall, 1997; Grabowski et al., 2001; Yoon et al., 2002).

Os dinoflagelados possuem diversas formas e mecanismos de obtenção de energia (Smayda, 1997; Anderson et al., 2002), sendo um grupo taxonômico composto por organismos autotróficos, algumas espécies estritamente heterotróficas (Hallegraeff & Lucas, 1988) e outras que podem ser mixótrofica (Hansen, 2011; Flynn et al., 2013). Estas últimas possuem plastídeos com pigmentos fotossintéticos que lhes permitem utilizar fontes de carbono inorgânico (autotrofia), alternadas ou complementadas com fontes orgânicas dissolvidas ou, ainda, presas vivas (heterotrofia) por meio da fagocitose (Hansen, 1991; Reguera et al., 2012). Neste sentido, as interações intraespecíficas (competição) e interespecíficas (predação) tem relevante contribuição na estrutura ecológica do plâncton (Mitra et al., 2014). Para a obtenção de presas, os dinoflagelados podem: **a)** absorvê-las através de alguma fissura celular, como utilizado pelo dinoflagelado *Noctiluca scintillans*; **b)** capturá-las por intermédio de alguma substância produzida e exudata (adesiva, anestésica, tóxica, etc), onde a célula predadora envolve a presa e, em muitos casos, esta substância pré-digere o alimento (véu de alimentação), como observado no gênero *Protoperidinium* e, mais recentemente, no gênero *Goyaulax*; e **c)** fazer uso de mizocitose, um mecanismo que consiste na perfuração da presa, seguido de sucção de seu conteúdo intracelular (seiva citoplasmática) por intermedio de um pedúnculo alimentar, como utilizado pelo

gênero *Dinophysis* (Jacobson & Andersen, 1994; Nishitani et al., 2005; Mafra et al., 2016).

Em geral exibem uma baixa taxa de crescimento populacional, e uma das principais características ecológicas dos dinoflagelados, está na capacidade de realizar migrações verticais na coluna d'água (Doblin et al., 2006), e os modos alternativos de nutrição, especialmente a mixotrofia, que podem lhes conferir uma vantagem competitiva em relação a outros organismos planctônicos, sobretudo em situações desfavoráveis aos grupos comumente dominantes de microalgas (Burkholder et al., 2008). Devido a estas características, dinoflagelados tornam-se frequentemente dominantes sob condições de estabilidade meteorológica, notavelmente quando a coluna d'água apresenta-se estratificada (Wyatt, 2014). O elevado poder de locomoção dos dinoflagelados lhes permite buscar condições ambientais mais favoráveis ao crescimento e auxilia na aquisição direta ou indireta de nutrientes, o que pode envolver, inclusive, a formação de densos agregados de células para a captura e ingestão de outros organismos (Zingone & Enevoldsen, 2000; Smayda & Reynolds, 2001).

Dinoflagelados também exibem diferentes estratégias de sobrevivência, aquisição de recursos (orgânicos e inorgânicos), e competição interespecífica, seja através de um crescimento populacional mais intenso ("*bloom*"; Wyatt, 2014), ou por meio de mecanismos que minimizem suas perdas por herbivoria, como a produção de mucilagem (Vila et al., 2001), toxinas (Hernández et al., 1998) e substâncias alopáticas (Granéli et al., 2008). Como consequência, tais estratégias frequentemente resultam em elevada biomassa, alterando a cor, odor e demais aspectos sanitários da água (Yin et al., 2008). Quando esses processos ecológicos ocorrem associados a algum tipo de efeito deletério ao homem, à fauna aquática ou ao ambiente, seja de forma direta ou indireta, o evento recebe a denominação de Floração de Alga Nociva (FAN), fenômeno popularmente conhecido como "maré vermelha" (Hallegraeff, 1995).

Os efeitos negativos de uma FAN, associados à produção de compostos tóxicos pela microalga, podem incluir desde o decréscimo nas taxas alimentares (Buskey, 2008), reprodutivas e de crescimento de organismos marinhos contaminados (Tillmann et al., 2008), até a morte ou a intoxicação de seus consumidores (Hernández et al., 1998; García et al., 2004; Friedman et al., 2017). A virulência e a magnitude de uma FAN variam conforme a espécie ou

grupo taxonômico envolvido, do modo de ação do composto tóxico produzido, o tipo de organismo exposto ao evento (ex. moluscos, crustáceos, peixes, aves, mamíferos) e das condições ambientais locais, que determinam a dimensão espacial e temporal do evento e dos impactos associados (Zingone & Enevoldsen, 2000; Granéli et al., 2006; Smayda, 2008; Glibert, 2016).

Casos de intoxicações entre os consumidores de pescados contaminados figuram como a principal preocupação de ordem socioeconômica associada aos impactos negativos das FANs, (FAO/WHO, 2016). Dinoflagelados são responsáveis pela maioria das intoxicações alimentares decorrentes do consumo de organismos marinhos contaminados por toxinas, incluindo a Ciguatera, e os envenenamentos neurotóxico, paralisante e diarreico por consumo de moluscos – NSP, PSP e DSP, respectivamente. Espécies de dinoflagelados produtores de toxinas paralisantes, como *Gymnodinium catenatum* (Proença et al., 2001; Negri et al., 2007) e *Alexandrium* spp., (Anderson et al., 1990; Persich et al., 2006) e de toxinas diarreicas, como *Prorocentrum lima* (Proença et al., 1999) e diversas espécies do gênero *Dinophysis* (Schmitt *&* Proença, 2000; Haraguchi *&* Odebrecht, 2010; Mafra et al., 2015a; Tibiriçá et al., 2015) têm sido reportadas no litoral sul do Brasil, por vezes provocando efeitos deletérios como observados durante florações de *Dinophysis* cf. *acuminata* (Proença et al., 2007; Mafra et al., 2015b)

O gênero *Dinophysis* (Ehrenberg) agrupa dinoflagelados tecados com morfologia celular peculiar. Além da assimetria entre as placas tecais (epiteca e hipoteca) que compõem sua carapaça celulósica externa, a forma, número e dimensões destas placas e das aletas (ou costelas) são características determinantes para a taxonomia das espécies deste gênero (Steidinger and Jangen, 1997; Faust & Gulledge, 2002). A principal e mais clássica forma de identificação taxonômica das espécies de *Dinophysis* se dá por meio da análise microscópica das características celulares. Contudo, o polimorfismo, relacionado com os diferentes estágios do ciclo de vida e como resposta às variações do ambiente, geram frequentes problemas taxonômicos, produzindo variações intraespecíficas ou semelhanças interespecíficas (Bravo et al., 1995; Reguera & Gonzalez-Gil, 2005). Algumas ferramentas e métodos de análises moleculares, como a transcrição da subunidade pequena do DNA ribossomal (18S), vêm sendo empregadas na construção da filogenia deste gênero. Entretanto, ainda

permanecem importantes lacunas de conhecimento acerca da variabilidade genética observada entre as populações (Edvardsen et al., 2003; Raho et al., 2008; Edvardsen et al., 2013; Stern et al., 2014).

Dez dentre as mais de cem espécies descritas de *Dinophysis*, além de duas espécies recentemente transferidas para o gênero *Phalacroma*, são capazes de produzir toxinas lipofílicas causadoras da síndrome diarreica – DSP (*Diarrheic Shellfish Poisoning*) – em humanos (Lee et al., 1989; Reguera et al. 2012). Estas toxinas vêm sendo detectadas em diversos países (revisto em Reguera et al., 2014), inclusive no sul do Brasil (Proença et al., 2007; Mafra et al., 2013, 2015b), causando danos à saúde pública, além de prejuízos para a indústria aquícola e pesqueira por meio da contaminação de pescados (e.g., Morgan et al., 2009; Sar et al., 2010).

Parte das populações neríticas de *Dinophysis* spp. ocorrem em ambientes costeiros abrigados, como enseadas e estuários (Koukaras, 2004; Aissaoui et al., 2013), onde pressupõem-se que tenham sua distribuição regulada por condições abióticas específicas, tais como a fraca turbulência da água (maior estabilidade), baixa salinidade, presença de fontes orgânicas e inorgânicas de nutrientes como o nitrogênio (Hattenrath-Lehmann et al., 2015), além de aspectos ecológicos como a interação trófica com os demais componentes microplanctônicos, evidenciada principalmente pela co-ocorrência de populações de *Dinophysis* e de sua supostamente exclusiva presa, o ciliado cleptoplastídico *Mesodinium rubrum* (Rial et al., 2012; Hansen et al., 2013).

Ambientes estuarinos sofrem grandes pressões antrópicas (Popovich & Marcovecchio, 2008; Artigas et al., 2014; Hattenrath-Lehmann et al., 2015) e neles importantes processos biogeoquímicos contribuem para o equilíbrio e manutenção de ecossistemas associados a zona costeira (Wyatt, 2014), contribuindo com a ciclagem compostos orgânicos inorgânicos (Dugdale et al., 2013), e com a exportação de material orgânico (particulado e dissolvido) para os ambientes costeiros adjacentes (Wolanski et al., 2008). Justamente nestes locais encontram-se as maiores diversidades de organismos e as mais intensas interações intraespecíficas (Gypens et al., 2013) e interespecíficas (Sohma et al., 2008) entre as espécies marinhas, principalmente do plâncton, que normalmente se distribuem ao longo de um gradiente termo-halino (Ferreira et al., 2005), geoquímico e/ou hidrodinâmico (Cloern, 1987; Acha et al., 2008).

Dinoflagelados do gênero *Dinophysis* no sul do Brasil

No Sul do Brasil espécies de *Dinophysis* vem sendo registrada desde a década de 1990, principalmente em ambientes costeiros como estuários, baías e enseadas (Proença, 1998; Schmitt & Proença, 2000; Mafra et al., 2006; Haraguchi & Odebrecht, 2010; Tibiriçá et al., 2015). Embora a presença de *Dinophysis* seja recorrente (Tavares et al., 2009; Proença et al., 2011), ainda permanece pouco explorado o conhecimento sobre quais são as principais condicionantes ambientais relacionadas à presença destes dinoflagelados. Parte do conhecimento ecológico sobre o gênero *Dinophysis* advém de estudos realizados em ambientes estuarinos costeiros mais ou menos profundos, sobretudo fiordes, que apresentam padrões hidrodinâmicos bem definidos (Blanco et al., 2007). Nestes locais, a presença destes organismos relaciona-se com os fenômenos de ressurgência, estratificações da estrutura físico-química da água e a disponibilidade de presas (Bravo et al., 1995; Velo-Suárez et al., 2014; Reguera et al., 2014).

Nos estuários subtropicais, especialmente no Atlântico Sul, o conhecimento sobre a distribuição e a ecologia de *Dinophysis* spp. ainda permanece escasso, principalmente no que tange às variações na ocorrência e abundância em menores escalas de tempo (variações diárias e semanais) e de espaço (centímetros a metros na escala vertical; dezenas a centenas de metros na escala horizontal).

Considerando que *as condições abióticas afetam o comportamento populacional de Dinophysis spp., então alterações sazonais na temperatura do ar, pluviosidade e da salinidade em um estuário raso refletirão em variações temporais na ocorrência e abundância destes dinoflagelados, bem como na presença de toxinas lipofílicas em organismos suspensívoros (Hipótese 1).* Sob esta mesma premissa, surge ainda a hipótese adicional de que, *se as abundâncias das populações de Dinophysis spp. e de sua presa em um estuário raso estão associadas a diferentes níveis de circulação, disponibilidade de nutrientes inorgânicos, tais como nitrogênio (N) e fósforo (P), e de estabilidade da coluna d'água, que por sua vez, estão relacionadas às variações na profundidade e hidrografia da região estuarina, então espera-se encontrar uma*

variação horizontal em meso-escala (centenas de metros) na distribuição destes dinoflagelados e de suas toxinas nestas regiões (Hipótese 2).

Além da importância ecológica, as enseadas, baías e estuários são regiões costeiras de grande relevância econômica, seja devido aos diferentes usos industriais, comerciais e recreativos (Bricker et al., 2008; Bet et al., 2015). Devido à sua elevada produtividade biológica, sustenta um alto nível de produção de alimentos de origem marinha por meio de captura e cultivo (Jonge, 2000; Dumbauld et al., 2009; Pereira et al., 2013). O litoral do Estado de Santa Catarina está localizado próximo a uma das áreas mais produtivas do oceano Atlântico Sul (Bisbal, 1995; Gibb et al., 2000; Garcia et al., 2004). Situado na extremidade sul da Plataforma Continental do Sudeste (PCSE: 23°S - 28,5°S), o litoral catarinense abriga a maior frota pesqueira industrial do Brasil (Andrade, 1998; Schwarz & Perez, 2007; Dallagnolo et al., 2010), e sustenta uma consistente atividade aquícola marinha (SEBRAE/SC, 2010; Jacomel & Campos, 2014).

Caracterizado por suas a heterogeneidade geomorfológica do litoral centro-norte catarinense promove as condições ideais para a instalação e operação dos cultivo de moluscos bivalves (Ferreira et al., 2007). Desde 1990, praticamente todos os municípios litorâneos implantaram unidades de produção de moluscos bivalves, com o predomínio de sistemas de gestão familiar (Novaes et al., 2011). A aquicultura marinha em Santa Catarina representa uma importante atividade econômica, geradora de emprego e renda, que se estrutura basicamente a partir do cultivo de mexilhões (*Perna perna*), ostras (*Crassostrea gigas*) e vieiras (*Nodipecten nodosus*), além da extração de moluscos endopsâmicos como o berbigão ou *vôngole* (*Anomalicardia brasiliana*). Contribuindo com 95% da produção nacional de moluscos bivalves, o estado produziu, em 2016, 15.381,44 toneladas destes organismos, sendo cerca de 12.534,1 ton de mexilhões, 2.280,46 de ostras e 27 de vieiras. As principais áreas de produção são as regiões das baías Sul e Norte (grande Florianópolis) e baía de Tijucas, no litoral central, além da enseada da Armação do Itapocoroy (Penha) e da Baía da Babitonga (São Francisco do Sul), no litoral norte (Manzoni, 2005; Marenzi & Branco, 2005; Novaes et al., 2011; Santos & Costa, 2017).

Milhões de dólares são investidos anualmente, em todo o mundo, no monitoramento, controle e prevenção da contaminação de pescados destinados

ao consumo humano incluindo toxinas produzidas por algas (Donald et al., 2001; Pagis et al., 2006; Coco et al., 2007; Higman et al., 2007; Jin & Hoagland, 2008; Vale et al., 2008; Trainer & Hardy, 2015). De todo modo, estudos em diversas regiões do planeta têm comprovado a relação entre a presença de níveis alarmantes de toxinas causadoras de DSP em moluscos bivalves e a ocorrência de células de *Dinophysis* spp. na água do mar, por vezes em densidades celulares tão baixas quanto poucas centenas de células por litro (Pavela-Vrancic et al., 2002; Moroño et al., 2003; Swanson et al., 2010; Mafra et al., 2013; 2015; Reguera et al., 2014). Em Santa Catarina, as diatomáceas do gênero *Pseudo-nitzschia* e, sobretudo, os dinoflagelados do gênero *Dinophysis* são frequentemente encontrados sob variadas densidades celulares em áreas de cultivos de moluscos bivalves (Proença et al., 2011).

O primeiro programa de monitoramento de algas nocivas em áreas de produção de moluscos no Brasil teve início em 1997, no município de Penha em Santa Catarina, com uma abordagem científica exploratória (Rorig et al., 1998; Alves et al., 2010). Desde os primeiros registros que datam da década de 1990, a classificação taxonômica por microscopia ótica identificou a espécie *D. acuminata*, como sendo o responsável pela presença de toxinas causadoras de DSP no sul do Brasil (Proença, 1998). Um estudo recente durante uma grande floração de *Dinophysis* no Uruguai, mostrou uma maior similaridade com a espécies *D. ovum* (S. *M.* Méndez et al., 2016). Até o momento a distinção entre *D. acuminata* e *D. ovum* permanece indefinida, mesmo com os avanços nas análises genéticas e moleculares e desta forma neste estudo foi denominado o agrupamento *D. acuminata* + *D. ovum* como *D.* cf. *acuminata*, na qual pressupõem-se que tais populações possam co-existir no tempo e no espaço.

A legislação brasileira, trata *D. acuminata* como o principal agente causador de contaminação de pescados por toxinas causadoras de DSP e recomenda o método de análise microscópica de sedimentação em câmaras de Utermöhl para identificação e quantificação dos organismos na água de cultivo de moluscos bivalves. Após uma sequência de FANs durante o ano de 2007 (Proença et al., 2007b), o monitoramento das áreas marinhas de produção aquícola passou a ser executado pelo governo federal em parceria com órgãos estaduais. Desde 2012, com a publicação da Instrução Normativa Interministerial Nº- 7 (BRASIL, 2012a), que implantou o Programa Nacional de Monitoramento

Higiênico e Sanitário de Moluscos Bivalves (PNCMB), a produção de moluscos bivalves vem sendo oficialmente e regularmente monitorada no Estado de Santa Catarina. De forma complementar, a Portaria nº 204 (BRASIL, 2012b) estabeleceu os procedimentos para coleta de amostras visando à realização de análises, dentre as quais, de toxinas em moluscos bivalves e contagens celulares para o monitoramento de espécies de microalgas potencialmente produtoras de toxinas, recomendando o monitoramento do gênero *Dinophysis* como principal organismo produtor de toxinas causadoras de DSP.

Desta forma, s*e Dinophysis spp. são a principal fonte de toxinas diarreicas no ambiente de estudo, espera-se obter uma relação entre a concentração e a composição de toxinas lipofílicas sintetizadas por estas populações e aquelas acumuladas em organismos marinhos co-ocorrentes na região, durante eventos de proliferação massiva, ou de expressiva abundância destas populações (Hipótese 3).*

Ecologia de dinoflagelados do gênero *Dinophysis*

Até cerca de duas décadas atrás, considerava-se que o gênero *Dinophysis* era composto exclusivamente por organismos autotróficos (Hallegraeff & Lucas, 1988). No entanto, estudos mais recentes foram demonstrando a co-ocorrência destes dinoflagelados com outros grupos taxonômicos componentes do nano- e microplâncton no ambiente, particularmente com criptofíceas e protozoários ciliados (Minnhagen & Janson, 2006; Sjöqvist & Lindholm, 2011). Ademais, foram frequentemente documentadas células de *Dinophysis* spp. contendo vacúolos alimentares repletos de organismos, ou de organelas remanescentes, (Hansen, 1991; Jacobson & Andersen, 1994; Hackett et al., 2003; Janson & Granéli, 2003; Carvalho et al., 2008), bem como um perfil pigmentar compatível com outros táxons (e.g. Rial et al., 2012), o que sustenta a tese de mixotrofia dentre estes dinoflagelados. De fato, o subsequente estabelecimento de culturas de algumas espécies de *Dinophysis* através do cultivo consorciado com o ciliado *M. rubrum*, alimentado por sua vez de criptofíceas (*Teleaulax amphioxeia* ou *Geminigera cryophila*), demonstrou que *Dinophysis* spp. exibem comportamento mixotrófico mediante a disponibilidade de presas específicas, no caso *M. rubrum* (Park et al., 2006). Estes ciliados, depois de ingerirem organismos nanoplanctônicos,

geralmente criptofíceas, são capazes de reter seus plastídios e outros fragmentos celulares de modo que tais organelas permaneçam ativas, possibilitando ao ciliado gerar energia através da fotossíntese (Johnson et al., 2007). Assim, células de *Dinophysis* spp., ao predarem o ciliado por meio de mizocitose, roubam-lhes seus plastídios temporários (ou cleptoplastídios) e também usufruem de sua funcionalidade fotossintética por algum tempo, sendo este mecanismo denominado de cleptoplasia ou cleptoplastia (Sjöqvist *&* Lindholm, 2011; Stern et al., 2014).

Os plastídios encontrados em *Dinophysis* spp. podem ter origem em diferentes grupos taxonômicos, tais como criptofíceas e cianobactérias, corroborando a diversidade de presas usadas pelos ciliados capturados por *Dinophysis* (Koike et al., 2005; Minnhagen et al., 2008; Garcia-Cuetos et al., 2010; Qiu et al., 2011; Hansen et al., 2013; Kim et al., 2015). Apesar de dividirem sequências genéticas idênticas aos de suas presas (e.g. Nishitani et al., 2010), os plastídios de *Dinophysis* se diferem ultra-estruturalmente dos encontrados nos ciliados (e.g. Garcia-Cuetos et al., 2010), o que gerou, por um tempo, conclusões conflitantes a respeito da capacidade de *Dinophysis* spp. em sintetizar seus próprios plastídios permanentes. Kim et al., (2012) demonstraram que, após a ingestão, os cleptoplastídios são estruturalmente modificados e retidos temporariamente por *Dinophysis* spp., o que explicaria os resultados conflitantes de estudos anteriores. De fato, o crescimento de *Dinophysis* spp. em laboratório (cultivos) ocorre somente mediante a disponibilidade de presas (e.g. Mafra et al., 2014), sugerindo que não há síntese de cloroplastos próprios e indicando a mixotrofia por meio da aquisição constante de cleptoplastídios como uma condição indispensável ao crescimento de *Dinophysis* (Tong et al., 2010; Rial et al., 2012).

Considerando a cleptoplastia como um mecanismo trófico intrínseco ao ciclo de vida de *Dinophysis*, e que as populações de *Dinophysis* spp. co-ocorrem com outros táxons do plâncton que se agregam na coluna d'água, será testada a hipótese de que *existe*, *, uma variação em pequena escala (dezenas de centímetros) na abundância do dinoflagelado e uma correlação com a abundância de outros grupos taxonômicos ao longo de um perfil vertical na coluna d´água (Hipótese 4)*;

O conhecimento acerca do efeito de condições ambientais sobre o crescimento e fisiologia de microalgas normalmente ocorre por intermédio de experimentos de laboratório, os quais permitem manipular e controlar os diferentes parâmetros que afetam a taxa de crescimento e o desenvolvimento destes organismos. Os métodos para cultivo em laboratório de dinoflagelados marinhos, nocivos ou não, variam de acordo com a espécie, habitat, forma de nutrição e aquisição de energia, sendo desenvolvidos majoritariamente com organismos foto-autotróficos (Islabão & Odebrecht, 2015). Requisitos operacionais e logísticos muitas vezes limitam a execução e os resultados obtidos em experimentos baseados em cultivos envolvendo organismos microplanctônicos heterotróficos ou mixotróficos. Por outro lado, experimentos de incubação de amostras de plâncton por períodos curtos (e.g. Marshall et al., 2005; Yin et al., 2008; Tong et al., 2010) podem prover conhecimentos valiosos sobre a relação trófica e a ecologia da comunidade microplanctônica, incluindo a possibilidade de se manipular fatores abióticos influentes. Assim, *se a estratificação da coluna d'água e a irradiância, afetam a distribuição vertical em pequena escala das populações de Dinophysis spp. e de suas presas, e se a migração vertical e/ou agregação de células fazem parte de um comportamento predatório em Dinophysis spp., então espera-se encontrar uma agregação do dinoflagelado e de sua presa em determinadas camadas da coluna d'água ao longo do dia, e em resposta a manipulações no grau de estratificação térmica da coluna d'água em um ambiente controlado de laboratório (Hipótese 5).*

OBJETIVOS

Objetivo Geral

Investigar, em diferentes escalas temporais e espaciais, a distribuição dos dinoflagelados do gênero *Dinophysis* (Ehrenberg) no litoral norte de Santa Catarina (SC), avaliando aspectos de sua ecologia populacional, suas relações tróficas e produção de toxinas lipofílicas, bem como o acúmulo destas toxinas em diferentes componentes da fauna marinha, com ênfase em moluscos bivalves.

Objetivos Específicos

I. Determinar os períodos do ano com maior risco para a ocorrência de toxinas diarreicas, com base em informações geradas ao longo de uma série de dados de oito anos, relacionando a abundância das populações de *Dinophysis* spp. à presença de toxinas diarreicas acumuladas em moluscos bivalves cultivados na Baía da Babitonga, no litoral norte de SC;
II. Determinar as condições abióticas que condicionam mais fortemente a ocorrência e abundância das populações de *Dinophysis* spp. e de suas presas ao longo do tempo na Baía da Babitonga, um estuário subtropical raso e bem misturado, com frequente ocorrência do dinoflagelado;
III. Comparar a presença e abundância de *Dinophysis* spp. e de suas presas entre duas áreas com hidrodinâmica e profundidades distintas na Baía da Babitonga, permitindo inferir sobre a influência desta característica do ambiente na dinâmica das populações ao longo do tempo e do espaço;
IV. Documentar a presença de toxinas lipofílicas e quantificar suas concentrações em moluscos bivalves, em células de *Dinophysis* spp. e na fração particulada da água do mar na Baía da Babitonga, avaliando quais condições abióticas e de disponibilidade de presas são mais favoráveis à produção de toxinas e ao seu acúmulo por organismos suspensívoros;
V. Avaliar as relações tróficas entre *Dinophysis* spp. e outros componentes da comunidade microplanctônica, com especial atenção às microalgas criptofíceas e protozoários ciliados, através da correlação entre suas abundâncias (por microscopia), da variação na composição de pigmentos

fotossintéticos e no perfil e concentração de toxinas lipofílicas (ambos por HPLC) ao longo do tempo e em diferentes profundidades, durante uma floração massiva de *Dinophysis* spp. na Enseada da Armação do Itapocoroy em Penha/SC;

VI. Avaliar, por espectrometria de massas (LC- MS/MS), o acúmulo e o perfil de toxinas lipofílicas nos diferentes componentes da fauna marinha durante o desenvolvimento de uma floração massiva de *Dinophysis* spp. na Enseada da Armação do Itapocoroy em Penha/SC;

VII. Investigar, sob condições controladas de laboratório e em escala temporal e espacial mais reduzida, a interação ecológica entre *Dinophysis* spp. e outros organismos planctônicos em resposta à variações de temperatura e estabilidade da coluna d'água.

ESTRUTURA DA TESE

A tese está estruturada na forma de quatro capítulos, contemplando abordagens metodológicas e escalas complementares. Inicia com (i) a investigação sobre aspectos ecológicos associados a padrões de grande escala de tempo (oito anos), e segue com (ii) o detalhamento de aspectos ecológicos em meso-escala, de tempo (um ano) e espaço (centenas de metros), (iii) o refinamento adicional para questões relacionadas às menores escalas, como variações diárias ao longo de poucos metros de profundidade durante uma floração massiva. Finalizando com (iv) a incubação experimental de amostras naturais com a finalidade de se observar o comportamento ecológico destes dinoflagelados em micro-escalas (centímetros e horas), também contribuiu com informações relevantes para este estudo.

O Capítulo 1 tem como enfoque as variações interanuais, na abundância e na toxicidade associada à ocorrência de *Dinophysis* spp. em uma área de produção de moluscos bivalves na Baía da Babitonga, entre os anos de 2007 e 2015. Esta abordagem contribuirá para o conhecimento dos períodos de maior incidência na ocorrência de *Dinophysis* spp. no litoral catarinense, bem como sobre a relação entre a densidade populacional e o acúmulo de toxinas em bivalves, atendendo ao objetivo específico I da tese. Foi publicado na revista *Environmental Monitoring and Assessment* (Springer), com fator de impacto (FI) >1.6 e classificação Qualis B2 para a área de Biodiversidade da CAPES. Um

resumo destes resultados foi também apresentado na *International Conference on Harmful Algae* (ICHA 2016), em outubro de 2016.

No segundo Capítulo, a abundância e a toxicidade de *Dinophysis* spp., assim como o perfil de toxinas lipofílicas acumuladas em duas espécies de moluscos bivalves, são investigados com uma frequência amostral quinzenal ao longo de 13 meses (fevereiro de 2015 a fevereiro de 2016). As variáveis são relacionadas com as condições abióticas e abundância dos demais táxons da comunidade planctônica em duas áreas com profundidade e hidrodinâmica distintas na mesma região produtora de moluscos bivalves na Baía da Babitonga (objetivos específicos II - IV). O manuscrito que compõem este capítulo será submetido para publicação na revista *Journal of Shellfish Research*, (classificação Qualis B1 na área de Biodiversidade da CAPES e FI >0,8). DA mesma forma que o capítulo anterior, um resumo dos resultados preliminares deste capítulo também foi apresentado na *International Conference on Harmful Algae* (ICHA 2016), em outubro de 2016.

No capítulo 3, através de um delineamento amostral mais refinado, buscou-se determinar os padrões de variação em pequena escala espacial e temporal na ocorrência e distribuição dos organismos planctônicos nos diferentes estratos verticais da coluna d'água, durante uma floração massiva de *Dinophysis* spp. (objetivo específico V). Este evento ocorreu entre o final de maio e início de junho de 2016, atingindo toda região sul do Brasil e parte da região sudeste; as amostragens relacionadas a este capítulo foram realizadas na Enseada de Armação do Itapocoroy, em Penha, um dos locais mais afetados pelo evento tóxico em SC. Foram investigados, além da interação entre *Dinophysis* e *M. rubrum*, a distribuição de toxinas lipofílicas na fração particulada do material em suspensão e acumulada em organismos representantes de diferentes níveis tróficos (objetivo específico VI). Devido à relevância dos resultados obtidos neste capítulo, os resultados foram publicados na edição especial da revista Toxins (Qualis A1 na área de Biodiversidade da CAPES e FI >3).

No capítulo 4, um experimento controlado em laboratório simula, em uma coluna d'água de um metro de profundidade, uma condição de estabilidade da água associada com diferentes níveis de estratificação térmica da água. Este procedimento teve como objetivo manipular determinadas condições abióticas a

fim de se observar, principalmente a resposta das populações de *Dinophysis* spp., e de suas presas potenciais, *M. rubrum*, além de outros organismos fitoplanctônicos em uma escala de centímetros e horas (objetivo específico VII). Este capítulo, terá a *Journal of Plankton research* (Qualis A2 na área de Biodiversidade da CAPES e FI >1,8) como revista-alvo pretendida para submissão deste artigo. A elaboração do capítulo foi realizada no formato de artigo científico necessitando ainda ser revisado pelos autores, ser traduzido para o idioma inglês e então ser enviado à um revisor/editor e posterior submissão.

Por fim, uma seção de Considerações Finais trará uma síntese integrativa dos resultados, abordando e avaliando em sinergia as variações nas diferentes escalas no tempo e no espaço, com vistas a uma gestão mais segura e sustentável da atividade aquícola em regiões afetadas por episódios de DSP. As referências bibliográficas citadas ao longo de toda a tese estão listadas em uma seção única, ao final do documento. Os ANEXOS I (prancha taxonômica) e II (ConCel), compõem as informações suplementares desta Tese, apresentado logo após a seção de referências.

CAPÍTULO 1 - Interannual variability in *Dinophysis* spp. abundance and toxin accumulation in farmed mussels (*Perna perna*) in a subtropical estuary.

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Abstract

This study evaluated an eight-year dataset (2007 to 2015, except 2008) in the attempt to identify the most susceptible periods for the occurrence of diarrheic shellfish poisoning (DSP) episodes associated with the presence of toxigenic dinoflagellates, *Dinophysis* spp., in the mussel-farming area of Babitonga Bay (southern Brazil). *Dinophysis* cf. *acuminata* was the most frequent (present in 65.7% of the samples) and abundant (max. 4100 cells L^{-1}) species, followed by *D. caudata* (13.8%; max. 640 cells L^{-1}) and *D. tripos* (0.9%; max. 50 cells L^{-1}). There was a marked onset of the annual rise in *Dinophysis* spp. abundance during weeks 21–25 of each year (early winter), followed by a second peak on week 35 (spring). Mussel (*Perna perna*) samples usually started testing positive in DSP mouse bioassays (MBA) in week 33 (August). Positive results were more frequent in 2007 and 2011, when the mean *D.* cf. *acuminata* abundance was ~500 cells L^{-1}. Although positive DSP-MBA results were observed in only 10.6% of the samples during the studied period, the toxin okadaic acid (OA) was present in 90% of analyzed mussels (max. 264 µg Kg^{-1}). MBA results became positive when *D.* cf. *acuminata* cell densities exceed 1200 ± 300 cells L^{-1}, while trace amounts of free and conjugated OA could be already detected at cell densities as low as 150 ± 50 or 200 ± 100 cells L^{-1}, respectively. Low salinity and the meteorological conditions triggered by La Niña events were the main factors associated with both *Dinophysis* abundance and OA accumulation in mussels in this area.

Keywords: DSP, Lipophilic toxins; Toxic dinoflagellates; HAB monitoring; Bivalve aquaculture.

Introduction

Okadaic acid (OA) and *Dinophysis*toxins (DTX) are the metabolic byproducts of marine dinoflagellates responsible for diarrhetic shellfish poisoning (DSP) in humans. These compounds affect negatively the shellfish production chain, demanding constant monitoring by regulatory agencies in order to assess public health-related risks (Lee et al. 1989; Reguera et al. 2012). High toxicity to humans associated with relatively low abundances of dinoflagellates belonging to the genus *Dinophysis* – producers of lipophilic toxins such as OA, DTX and the non-diarrheic pectenotoxins (PTX) – places DSP as one of the most serious issues concerning the safety of seafood consumption worldwide (Higman et al. 2007; Nelan 2007; Silke 2008).

Over the last decade, the detection of diarrheic toxins and their producing microalgae has increased around the globe, imposing negative impacts on public health and important regional economic losses in different regions (Brahim, 2007; Morgan et al., 2009) including developing countries in South America, as reviewed by Reguera et al. (2014). Several *Dinophysis* species and their related toxins have been reported in the South American continental shelves, mainly in the subtropical coasts of Chile (Díaz et al. 2011; Trefault et al. 2011; Alves-de-Souza et al. 2014); Argentina (Sar et al. 2012; Sunesen et al. 2014; Turner and Goya 2015), Uruguay (Martínez and Ortega 2007; Martínez et al. 2017), and southern Brazil (Proença et al. 2007; Haraguchi and Odebrecht 2010; Mafra et al. 2014, 2015). Only in the southernmost coast of Brazil, 43 species of Dinophysiales were identified, including five classified as potentially toxic (*Dinophysis acuminata*, *D. fortii*, *D. ovum*, *D. caudata*, and *D. tripos*) (Haraguchi and Odebrecht, 2010).

Like in other South American regions, blooms of *Dinophysis* spp. have been frequently registered along the southern Brazilian coast, especially in Santa Catarina State (SC). During these events, cells of *Dinophysis* spp. – usually belonging to the *D. acuminata* taxonomic complex (*D. acuminata* + *D. ovum*) – can reach relatively high abundance values (10^3–10^4 cells per liter) and be associated with the presence of diarrheic toxins in marine organisms (Schmitt and Proença, 2000; Tavares et al., 2009; Proença et al., 2011). Therefore, in order to protect consumer health, managers have enforced harvesting closures to shellfish farming areas any time the regulatory limit of 160 µg OA equivalent

Kg^{-1} is surpassed, which has resulted in significant economic losses for mussel and oyster growers.

Many countries experiencing recurrent harmful algal blooms (HABs) employ management strategies, monitoring programs, and surveillance systems as tools to ensure safe seafood consumption (Anderson et al. 2001; Higman et al., 2007; Nelan, 2007; Vale et al., 2008; Wong et al., 2009; Lewitus et al., 2012; Li et al., 2012; Armi et al. 2012). In South America, the governments of Argentina, Uruguay and Chile have conducted well-established monitoring programs in aquaculture areas generating comprehensive and reliable information that can be readily assessed by decision-making managers during toxic bloom episodes (Fux et al., 2011; Turner and Goya, 2015; Martínez et al. 2017). In Brazil, the knowledge about the occurrence of HABs and their associated impacts is recent, scarce, and limited, especially outside the scientific community. Considering the lack of continuous information on the occurrence of HAB species and their related toxins, this study evaluated an eight-year dataset as a first attempt to identify the most susceptible periods for the occurrence of DSP episodes in an estuarine shellfish farming area in southern Brazil, representative of the environments where bivalve mollusks are typically cultivated in the country.

Material and methods

Study Area

Babitonga Bay (BB) is the largest estuarine area in Santa Catarina, Brazil, covering 1567 Km^2 of water surface, surrounded by a relatively pristine mangrove formation. This estuary is shallow (~6.0 m deep) and well mixed, dominated by a semi-diurnal micro-tidal cycle, and located in a wet subtropical zone with a mean annual precipitation of 2300 mm (Truccolo and Schettini, 1999). This highly hydrodynamic system produces a relatively large and well-developed tide delta, where Paulas – the most productive of the three main shellfish farming areas – is located (Vieira et al., 2008).

Altogether, shellfish farms produce 100 ton of mussels and 40 tons of oysters per year in BB, mainly employing suspended long line culture systems. Like in other farming areas in SC, which currently produces 95% of the national

20,438-ton bivalve production (Santos and Costa, 2015), bivalve aquaculture in BB is run by several low-income families mostly led by former fishermen (Ferreira et al., 2004; Jacomel and Campos, 2014). Shellfish harvesting bans linked to HAB events have therefore caused relevant economic impacts on this small-scale activity over the past decade, eventually forcing the government to pay compensations during long periods of shut down.

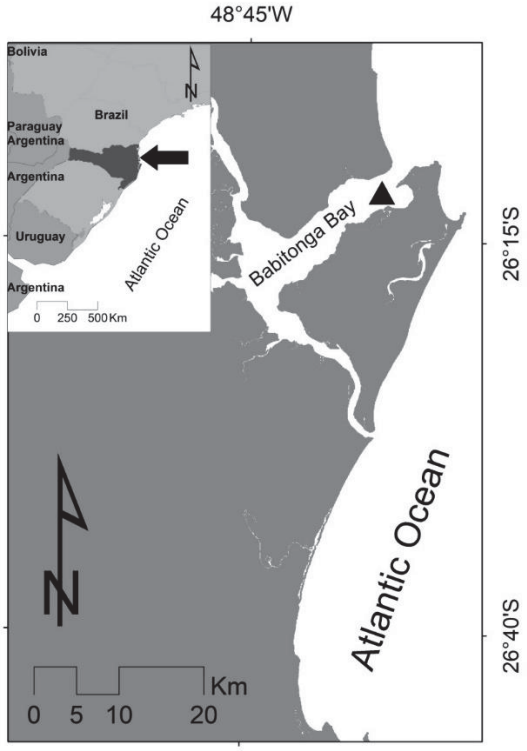

Figura 1 - Cap 1: Fig. 1 – The study area in Babitonga Bay, Santa Catarina State, southern Brazil. The solid black triangle indicates the location of the shellfish farming area in Paulas.

Sampling

The data set reported herein was generated under the scope of different monitoring efforts conducted from 2007 to 2015 (except 2008) in Paulas, BB (26.2 °S; 48.6 °W). Between 2007 and 2012, the occurrence of HAB species and their toxins was monitored by two different governmental agencies ("*Empresa Catarinense de Pesquisa Agropecuária e Extensão Rural de Santa Catarina – EPAGRI*", and "*Companhia Integrada de Desenvolvimento Agrícola de Santa Catarina – CIDASC*"). After 2012, these activities were incorporated into the new National Program for Molluscan Shellfish Hygienic and Sanitation Control

(BRASIL, 2012a), which maintained a similar sampling design and strategy, and analytical methods.

The bimonthly sampling included the collection of integrated water column samples using a 5-m hose, and sixty shellfish individuals taken from a randomly chosen long-line within the Paulas farming area. In addition, concentrated plankton samples were collected with a 20-µm plankton net obliquely dragged at ~0.5 m s^{-1} for two minutes from the bottom to the surface. Salinity was measured in the laboratory using a handheld YSI® professional plus multi parameter meter.

Meteorological data

Meteorological data were obtained from the National Institute of Meteorology (INMET, 2017), which keeps an extensive network of stations generating information that is made available to the public. Daily measures from three meteorological stations located to the south (Florianópolis-SC: 27.6 °S; 48.6 °W), west (Indaial-SC: 26.9 °S; 49.2 °W) and north (Ilha do Mel-PR: 25.5 °S; 48.3 °W) of the BB estuary were used to calculate the weekly and annual average values of air temperature and humidity, wind speed, and accumulated rainfall. In addition, the El Niño Southern Oscillation (ENSO) index was obtained from the online database provided by the Climatic Prediction Center of the National Weather Service from the US National Oceanic and Atmospheric Administration (NOAA, 2017).

Phytoplankton quantification

Each integrated water sample was fixed with Lugol's iodine solution and used for cell counting under an inverted microscope after settling an aliquot (10-20 mL) on a counting chamber according to the Utermöhl's method (Edler and Elbrächter, 2010)The total abundance of the micro-phytoplankton assemblage was recorded and expressed as cell L^{-1}, and taxa were classified within large taxonomic groups. *Dinophysis* species were identified based on their morphology under optical microscopy (Raho et al., 2008b; Reguera et al., 2012) and counted individually. Unfortunately, recent genetic analyses (based on ITS and Cox1 sequences) on cells isolated from the BB (not shown) have not allowed

discriminating the species within the *D. acuminata* taxonomic complex. The dominant species in this study will be, therefore, referred to as *Dinophysis* cf. *acuminata* based on microscopic observations.

Mouse Bioassay (MBA) for the detection of DSP toxicity

Acetone extracts for the DSP-MBA were prepared from mussel samples in accordance with the European harmonized method (Yasumoto et al. 1984; EURLM 2009). Positive results indicating the levels of diarrheic toxins above the regulatory limit for human consumption were attributed to the death of at least two out of three mice within 24 h following intraperitoneal toxin injection. Although the death of one out of three tested mice was considered a negative result, it might still indicate the presence of low levels of toxins. When this occurred, new mussel samples were taken a few days later for re-examination.

Toxin analysis by LC-MS/MS

In addition to the samples destined to the MBA, extra sample fractions composed of homogenized mussel tissues were available during the 2012-2015 period (n=71) for toxin characterization by liquid chromatography–mass spectrometry (LC-MS/MS). For that, 1.0 ± 0.5 g from each sample was mixed with 99.9% methanol at a 1:9 proportion (v:v) and exposed to an ultrasonic cell disruptor (130 W, Cole Parmer CPX130; USA) for 2 min (3 s pulses with 1 s intervals) at 80% amplitude for the extraction of lipophilic toxins. The homogenates were subsequently centrifuged at 2000 rpm for 10 min, and 1 mL of the supernatant was filtered through a PVTE syringe filter (0.22 µm) and collected into a glass vial for the LC-MS/MS analysis.

The toxin separation was conducted on an Agilent 1260 LC system using a C18 column (50×2.0 mm; 3 µm particle diameter) at 20 ºC. The mobile phases were composed of (A) 100% water and (B) 95% acetonitrile + 5% water, both with 2 mM ammonium formate + 50 mM formic acid. The gradient elution (0.3 ml min^{-1}) was applied as follows: from 80:20% (A:B) for 8 min, increased at 100% B,

and maintained for 3.5 min before returning to the initial condition until the end of analysis (13 min in total). Lipophilic toxins were detected in an AbSciex QTRAP3200 triple quadrupole mass spectrometer, with optimized parameters as described in Table 1 for OA, DTX-1, DTX-2 and DTX-3 (7-O-acyl-35-methyl OA). Free forms of these diarrheic toxins were screened upon the injection of 10- to 15-µL samples. The amounts of total toxin were determined in a second analysis after the alkaline hydrolysis of their metabolized conjugated forms in 1-mL aliquots of extracts (EURLMB, 2015). The concentrations of okadaic acid (i.e., free, conjugated, and total amounts) were calculated from a calibration curve generated from sequential dilutions of the analytical standard (3, 14, 56 and 223 ng mL^{-1}). The presence of other lipophilic toxins such as pectenotoxins (PTX-1, PTX-2 and PTX-6), yessotoxins (YTX) and their analogues (45-OH-YTX and Homo-YTX) was also monitored aiming at explaining possible MBA false-positive results (optimized MS parameters not shown).

Tabela 1 - Cap 1: Table 1 – MS/MS system configuration used to determine lipophilic toxins in mussel samples.

Toxins	ESI	Q1 (m/z)	Q3 (m/z)	mseg	DP (v)	EP (v)	CEP (v)	CE (v)	CXP (v)	Curtain Gas (CUR)	Collision Gas (CAD)	Voltage (IS)	Temp. (TEM)	Gas 1 (GS1)	Gas 2 (GS2)
OA	NEG	803.5	255.0	29	-129	-10	-40.1	-82	-2						
OA	NEG	803.5	113.0	29	-129	-10	-41.5	-64	-2						
DTX-2	NEG	803.5	255.0	36	-129	-10	-40.6	-64	-2						
DTX-2	NEG	803.5	113.0	36	-129	-10	-41.5	-84	-2	25 psi	Medium	4500 v	500 °C	40 psi	40psi
DTX-1	NEG	817.5	255.0	36	-129	-10	-41.5	-62	-2						
DTX-1	NEG	817.5	113.0	36	-120	-10	-51.7	-82	-2						
DTX-3	NEG	1041.6	255.0	36	-129	-10	-47.9	-76	-2						

Statistical analysis

The MBA results were expressed as numerical values considering the number of mice dead relative to the number of animals tested (0/3=0; 1/3=0.33; 2/3=0.67; 3/3=1), and was used in order to explore the relationships among the

MBA results, *Dinophysis* spp. abundances, OA contents, and the weekly averaged values of air temperature, wind speed, humidity, ENSO index and accumulated rain. Kruskal-Wallis test, followed by the Dunn's test, was used to identify significant differences ($p<0.05$) among weeks within years, and among years. Generalized additive models (GAM), with Poisson or Gama distribution and log-link function, were used to check the importance of environmental variables in explaining *Dinophysis* spp. abundance in the water and toxin occurrence in mussels. Four to 12 degrees of freedom (depending on each predictor) were selected in order to best fit the predictors (time of the year (weeks) or environmental variables) in response to the *Dinophysis* spp. cell density and toxin presence (from MBA) and concentration (from LC-MS/MS analysis). The abundance of *Dinophysis* spp. was also used as a predictor to evaluate the MBA results and the concentrations of OA (in both free and conjugated forms) in mussels. All analyses were performed in the Statistica 7.0® software (Version 7; StatSoft, Inc.).

Results

Periods of shellfish harvesting closures due to DSP risk (i.e., episodes of bivalve contamination with unsafe diarrheic toxin levels, as assessed by DSP-MBA) occurred at least six times throughout the sampling period, mostly between 2007 and 2011, including 2008 when no quantitative data were available from the monitoring programs. Furthermore, low to moderate OA concentrations were detected in a large proportion of the analyzed mussel samples (n=64/71) from 2012 to 2015, associated with the frequent occurrence of *Dinophysis* spp. (mainly *D.* cf. *acuminata*) in the water samples (Table 2).

Tabela 2 - Cap 1: Table 2 – Annual average values (± standard error) and sample number for each variable measured in BB from 2007 to 2015 (except 2008).

Variable/Year		2007	2009	2010	2011	2012	2013	2014	2015	Overall
DSP-MBA	Mean ±SE (n)	0.4 ±0.1 (23)	0.1 ±0.1 (20)	0.1 ±0.1 (22)	0.3 ±0.1 (38)	0.0 ±0.0 (24)	0.1 ±0.0 (35)	0.1 ±0.0 (32)	0.0 ±0.0 (33)	0.1 ±0.1 (227)
µg free-OA Kg^{-1}	Mean ±SE (n)					8.5 ±3.4 (11)	8.3 ±1.9 (13)	11.4 ±1.9 (24)	15.8 ±1.7 (23)	11.8 ±1 (71)
µg conj-OA Kg^{-1}	Mean ±SE (n)					64.1 ±11.0 (11)	49.0 ±13.7 (13)	55.4 ±13.6 (24)	71.9 ±8.4 (23)	60.9 ±7 (71)
µg total-OA Kg^{-1}	Mean ±SE (n)					72.6 ±24.1 (11)	57.4 ±15.5 (13)	66.7 ±15.2 (24)	87.7 ±8.5 (23)	72.7 ±7 (71)
D. cf. *acuminata* cells L^{-1}	Mean ±SE (n)	520 ±102 (24)	251 ±95 (26)	220 ±65 (25)	487 ±78 (39)	108 ±31 (26)	209 ±68 (37)	275 ±187 (22)	48 ±14 (33)	266 ±31 (232)
D. caudata cells L^{-1}	Mean ±SE (n)	79 ±29 (24)	6 ±4 (26)	ND (25)	74 ±24 (39)	ND (26)	4 ±3 (37)	ND (22)	ND (33)	22 ±5 (232)
D. tripos cells L^{-1}	Mean ±SE (n)	ND (24)	ND (26)	2 ±2 (25)	ND (39)	ND (26)	ND (37)	2 ±2 (22)	ND (33)	0.4 ±0.3 (232)

Salinity	Mean ±SE (n)	29 ±0.3 (8)	30 ±0.3 (26)	25 ±1.4 (5)	29 ±0.3 (10)	30 ±0.5 (20)	30 ±0.3 (34)	30 ±1 (22)	30 ±1 (33)	30 ±0.2 (158)
Air Temp (°C)	Mean ±SE (n)	19 ±1 (53)	21 ±1 (53)	21 ±1 (53)	19 ±1 (53)	21 ±1 (53)	21 ±1 (53)	22 ±1 (53)	22 ±1 (53)	21 ±0.3 (477)
Humidity (%)	Mean ±SE (n)	82 ±1 (53)	84 ±1 (53)	72 ±3 (53)	84 ±2 (53)	81 ±2 (53)	80 ±2 (53)	80 ±1 (53)	82 ±1 (53)	81 ±1 (477)
Wind speed ($m\ s^{-1}$)	Mean ±SE (n)	2.1 ±0.1 (53)	2.0 ±0.1 (53)	1.8 ±0.1 (53)	1.5 ±0.1 (53)	1.3 ±0.1 (53)	1.3 ±0.1 (53)	1.2 ±0.1 (53)	1.1 ±0.1 (53)	1.5 ±0.0 (477)
Accumulated rainfall (mm)	Mean ±SE (n)	1577 ±11 (53)	1990 ±16 (53)	2352 ±17 (53)	2344 ±18 (53)	1613 ±10 (53)	1726 ±13 (53)	1676 ±9 (53)	2436 ±12 (53)	1964 ±14 (53)

Environmental factors

In general, the meteorological-oceanographic variables exhibited annual average values (Table 2) and seasonal variation patterns (Fig 2A-D) within the usual range reported for this wet subtropical geographical region. The average (avg ± standard error (SE)) air temperature during the entire sampling period was 21 ± 0.3 °C, with slightly lower values recorded in 2007 and 2011 (~19- ºC). The lowest absolute value occurred in July of 2007 (11 °C) and the highest in February of 2013 (29 °C, Fig. 2A). Considering the weekly averaged values for the entire studied period, air temperature reached the maximum value on week 10 of each year (end of the southern hemisphere summer), decreasing thereafter until weeks 22–24 (late autumn), and remaining at minimum levels until weeks 30–32 (mid-winter) when it started to increase again towards the next warm season. An expressive annual temperature amplitude range (>25 ºC) was observed, with significantly higher (H= 189; p= *0.01*) values recorded at the beginning of the year compared to those registered in the middle of the year. However, no significant interannual variation was detected among yearly averaged values (H= 8.1; p= *0.42*). Air humidity did not vary significantly over the studied years (H= 56; p= *0.28*); although frequently elevated (annual avg= 81 ± 0.6%), values were slightly lower by the end of the year (Fig. 2B).

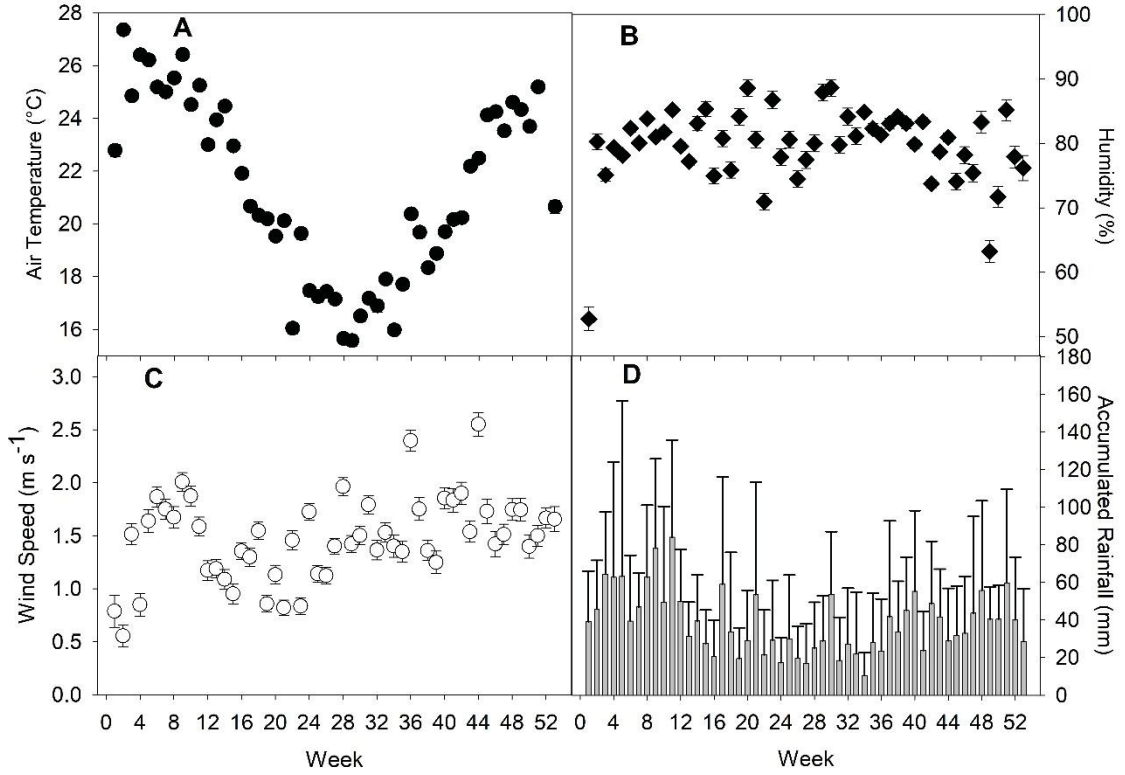

Figura 2 - Cap 1: Fig. 2 – Weekly average values (± standard error) for main meteorological variables measured during the investigated period (2007 to 2015, except 2008): A) air temperature (°C), B) air humidity (%), C) wind speed (m s^{-1}), and D) accumulated rainfall (mm). The week numbers follow the Gregorian calendar.

Most of the time, wind speed was below than 1.0 m s^{-1}, related to the trade winds of the northeast quadrant that act during late spring and early fall. In contrast, wind speeds >1.5 m s^{-1} weekly average, with wind gusts >10 m s^{-1}), as registered in winter-spring, indicates an increase in the frequency and intensity of cold fronts comes from southern with predominance of South quadrant winds. Considering the entire sampling period (Fig. 2C), low weekly wind speed average values (avg 1.0–1.5 m s^{-1}) were recorded in autumn (weeks 12–24), whereas values slightly higher than the overall mean (1.5 ± 0.1 m s^{-1}) occurred in winter and spring (after week 28), associated with strong atmospheric pressure gradients. At that time, long periods of atmospheric stability are usually disturbed by short periods (2–4 days) of strong southerly winds, thus increasing the average values. Even though, there were no significant differences among the weekly averaged wind speeds within a single year (H= 65; *p= 0.09*). On an interannual basis, there was a significant decrease in wind speed from 2007 to 2015 (H= 69; *p= 0.01*).

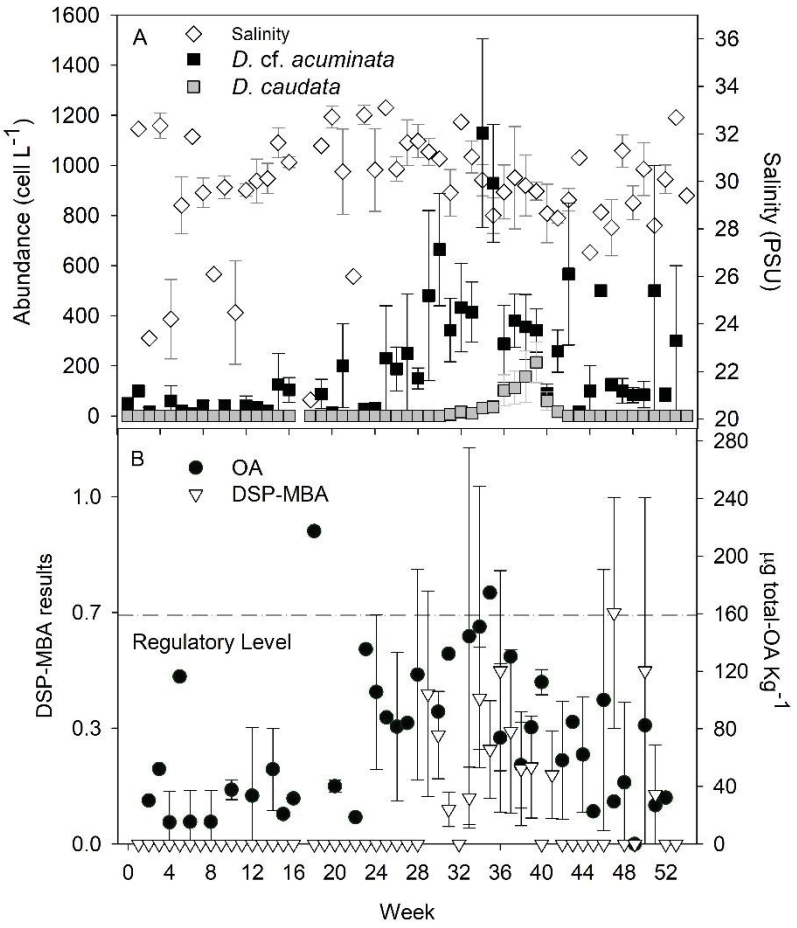

Figura 3 - Cap 1: Fig. 3 –Weekly averaged (A,B) values (± standard error) of (A) salinity (open diamonds), Dinophysis spp. cell abundance (squares), D. cf. acuminata (black square) and D. caudata (dark gray square); (B) DSP-MBA results (open inverted triangles), and okadaic acid (OA) concentration (solid circle) in soft tissues of brown mussels Perna perna along the investigated period (2007 to 2015, except 2008). The week numbers follow the Gregorian calendar.

Rainfall was frequently high during the sampling period (Fig. 2D), with a weekly accumulated average of 6 ± 0.2 mm, and peaks above 49 mm usually occurring from weeks 9 to 11 (late summer). Considering the entire period, there were no significant differences in rainfall among the weeks of each year (H= 15; *p= 0.06*). The mean annual precipitation for the entire period was 2066 ± 14 mm in this region, with values below the average in 2007 (1577 mm) and 2012 (1613 mm); and higher in 2010 (2352 mm), 2011 (2344 mm), and 2015 (2436 mm), which was the rainiest year.

Salinity ranged from 20 to 34 PSU (Fig. 3A-B) in Paulas (avg ± SE= 30 ± 0.2 PSU), demonstrating the influence of frequent freshwater discharge in this estuary, which may have contributed to a transient stratification of the water column (vertical and/or horizontal). In autumn, salinity values were up to 4 PSU

above the mean value, indicating a more prominent marine influence over the estuary during that period. Salinity was significantly lower (25 ± 3 PSU) in 2010 (Table 2) compared to all others investigated years (H= 21; *p= 0.01*).

Abundance of *Dinophysis* spp.

Dinophysis spp. was frequently found in water samples collected in Babitonga Bay (Fig. 3A), especially during autumn/winter. *D.* cf. *acuminata* was the most representative species, being present in 66% of the samples and occurring every year from autumn to spring and eventually in summer, at cell densities up to 4100 cells L^{-1}. *D. caudata* was less frequent (14%) and less abundant (maximum 640 cells L^{-1}), with occurrence restricted to winter/spring, while *D. tripos* occurred sporadically in 1% of the samples at cell densities ≤50 cells L^{-1}.

On average, *D.* cf. *acuminata* (avg ± SE: 266 ± 32 cells L^{-1}) contributed with 96% of the total number of *Dinophysis* cells in the water, occurring from week 18 to 52 of each year (Fig 3B). Cell densities were significantly high (H= 93; *p= 0.01*) during weeks 29 to 39. This taxon was more abundant in 2007 (520 ± 102 cells L^{-1}) and 2011 (487 ± 78 cells L^{-1}) when peaks of abundance >1000 cells L^{-1} persisted for at least 15 days, which also occurred in 2013. Conversely, the mean cell density of *D.* cf. *acuminata* was significantly low (H= 53; *p= 0.01*) in 2012, 2014, and especially in 2015, when maximum abundances were <300 cells L^{-1}. *D. caudata* was less abundant (22 ± 5 cells L^{-1}) and occurred slightly later during weeks 30-41 (Fig. 3B), associated with low salinity values (<28 PSU). Cell abundance of *D. caudata* was low (≤50 cells L^{-1}) in 2009 and 2013, and higher in 2007 (79 ± 29 cells L^{-1}) and 2011 (74 ± 24 cell L^{-1}) (H=68; *p= 0.01*), when values >500 cell L^{-1} were occasionally recorded. Finally, *D. tripos* occurred at levels above the quantification limit (50 cells L^{-1}) only in 2010 and 2014, exclusively at weeks 20 and 27 respectively.

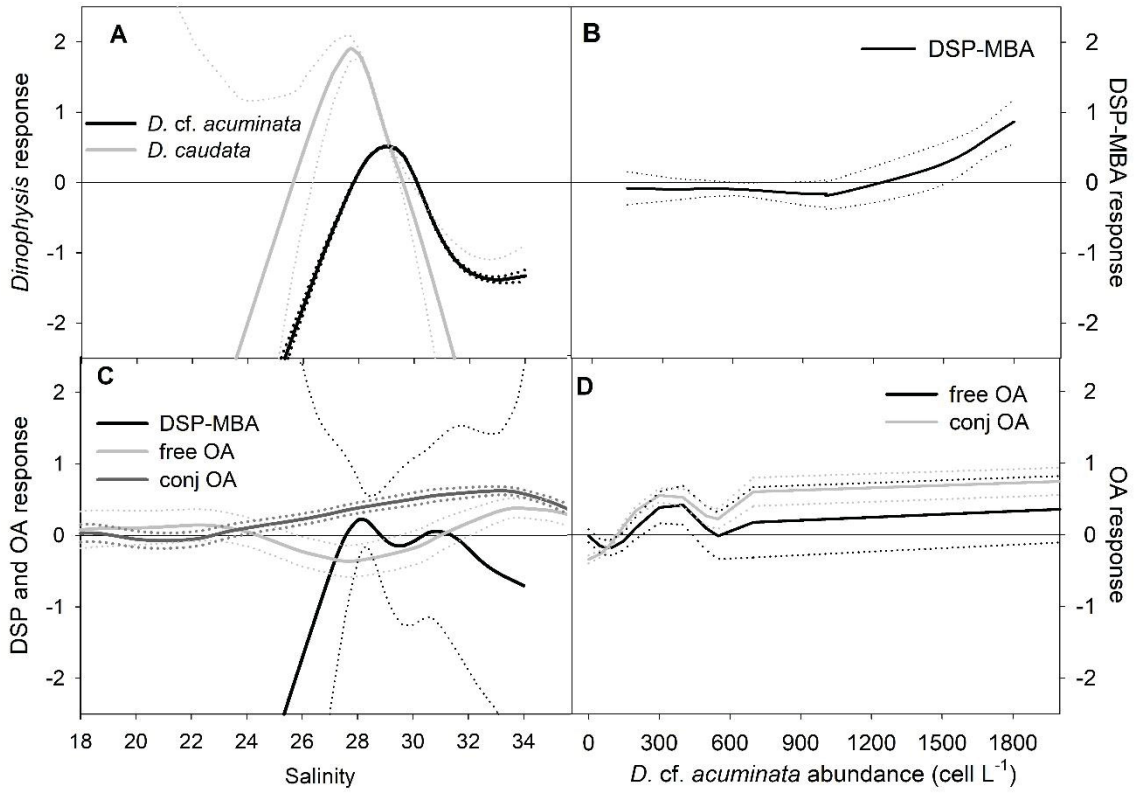

Figura 4 - Cap 1: Fig. 4 – GAM spline curves (solid colored lines) and 95% confident bands (dotted gray scale lines) expressing the anomalous residue values for (A) D. cf. acuminata and D. caudata cell abundance, and (C) DSP-MBA results, and concentrations of okadaic acid (OA) in the free and conjugated forms, in response to the following predictors: salinity. The point where the model line crosses zero in the Y-axis in B and D marks the threshold in D. cf. acuminata abundance for the initiation of positive DSP-MBA results (B) or for the detection of OA concentrations in mussels (D).

Diarrheic toxins and toxicity evaluated by MBA

Positive DSP-MBA results (Fig. 3B) occurred during most investigated years, except in 2012 and 2015. They were concentrated in two different periods of the year: weeks 29 to 39, with a peak of maximum frequency between weeks 33 and 36 (August-September), and weeks 45 to 52 (November-December) (Fig. 3B). Okadaic acid was detected in mussels (46 ± 44 µg total-OA Kg^{-1}) over a longer period in the year, typically from weeks 18 to 52 (Fig. 4D), and even in years when DSP episodes were less common, i.e., 2012–2015.

From 2012 to 2015, high OA levels coincided with the occurrence of positive DSP-MBA at two occasions in 2014, when high *Dinophysis* spp. cell abundances (max. 4100 cells L^{-1}) were detected in water samples, but not in 2012 when *Dinophysis* spp. were present at around 500 cell L^{-1} (Fig. 3A). Conversely, positive MBA results were reported twice in 2013 (October and November),

without the detection of correspondingly high OA levels in mussel tissues (only up to 25 µg total-OA Kg^{-1}) (Fig. 3B), nor of any trace of other lipophilic toxins such as DTX, PTX, or YTX. Around 11% of all samples tested positive in the MBA during the entire sampling period. Likewise, 11% of those tested by LC-MS/MS (2012-2015) contained OA concentrations above the regulatory limit (i.e., >160 µg total-OA Kg^{-1}. Unfortunately, no samples for toxin analysis were available previously to 2012, when DSP events were more frequent and severe (Table 2).

Free and conjugated (i.e., metabolized) forms of OA were simultaneously detected over the entire sampling period, on a ratio of ~1:4 (max. 35 and 140 µg Kg^{-1}, respectively). Levels of free-OA were approximately three times higher than the annual average during weeks 33-36, corresponding to the period when mussels also presented the greatest loads of conjugated toxin.

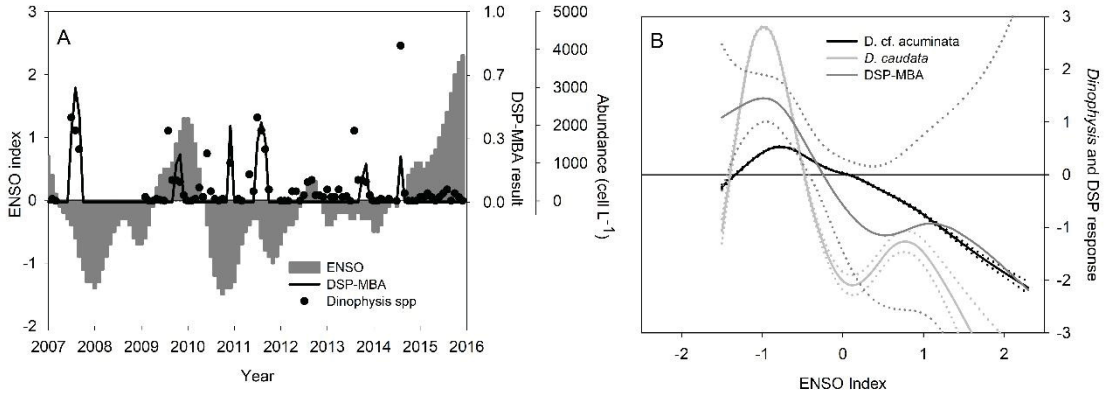

Figura 5 - Cap 1: Fig. 5 – (A) Discrete values of the El Niño Southern Oscillation (ENSO) index, abundance of Dinophysis spp., and positive DSP-MBA results along the investigated period (2007-2015); (B) GAM spline (solid line) and 95% confident band (dotted line) for D. cf. acuminata (black), D. caudata gray), and DSP-MBA (dark gray) as a function of the ENSO index.

GAM Analysis

The GAM cubic spline smoothing fit curves for the responses of *Dinophysis* spp. abundance values (Fig. 4A) indicate that *Dinophysis* spp. attain higher cell abundances at intermediate salinities than otherwise (28–30 PSU for *D.* cf. *acuminata* and 26–30 PSU for *D. caudata*). In addition, the models revealed that both the frequency of positive DSP-MBA results and the OA concentrations in mussels were also higher at intermediate salinities (Fig. 4C). More importantly, positive MBA results started to occur when *D.* cf. *acuminata* cell densities exceed 1200 ± 300 cells L^{-1} (Fig. 4B), while low to moderate amounts of free and

conjugated OA could be already detected in mussels at cell densities as low as 150 ± 50 or 200 ± 100 cells L^{-1}, respectively (Fig. 4D).

Monthly maximum *Dinophysis* spp. abundance values and average DSP-MBA results were both inversely related to the El Niño Southern Oscillation (ENSO) index (Fig. 5A). All DSP events occurred during La Niña conditions (so called LNSO; negative index), except in 2009 and 2014, when DSP events were associated with positive and neutral ENSO indices, respectively. Moreover, the more negative the index, the more persistent and severe the DSP events were, as assessed by the width and height of the MBA result peaks in Figure 5A, respectively. In fact, using ENSO as a predictor, GAM fitted curves (Fig. 5B) confirmed that periods under the effects of La Niña were more favorable to the occurrence of *Dinophysis* spp. than others, and that severe DSP outbreaks are not expected under the effects of El Niño (positive index).

Discussion

Toxigenic *Dinophysis* species have been recorded in the southwestern Atlantic coastal waters over the past decades (Schmitt and Proença, 2000; Mafra et al. 2006; 2015; Martínez and Ortega, 2007; Haraguchi and Odebrecht, 2010; Sar et al., 2012). Here, for the first time in Brazil, a comprehensive long-term analysis is presented aiming at describing the most favorable conditions driving the development of *Dinophysis* spp. – and the transfer of their toxins – in an estuarine area representative of this geographical region.

Dinophysis cf. *acuminata* was the most frequent and abundant *Dinophysis* taxon occurring in Babitonga Bay and the main responsible for the contamination of mussels with the diarrheic toxin okadaic acid (OA) during the study period. The highest cell abundance values (up to 4100 cells L^{-1}) were reported during the winter southern hemisphere months (July–September), with fewer cells being detected (from 50 to 200 cells L^{-1}) in autumn and spring. The driest periods, characterized by simultaneously decreasing air temperature and salinity, were associated with the highest abundance values of this dinoflagellate species and the occurrence of OA in bivalve mollusks. The detection of a second potentially toxigenic species, *D. caudata*, was more restricted to the spring months, and probably related to an increased influence of external marine waters in the

estuary. Nevertheless, what happens to these populations during the warmest season remains poorly documented.

In order to remain for prolonged periods (i.e. several months) inside the estuary, the obligate mixotrophic *Dinophysis* spp. would depend on the availability of its ciliate prey, *Mesodinium rubruM.* This common ciliate species can be found all year round along the southern Brazilian coast, including in coastal environments such as shallow bays, estuaries and lagoons (Odebrecht and Garcia, 1998). However, its highest abundances have been associated with weak winds and sunny weather (Owen et al., 1992), attaining up to 4.4×10^6 cell$\cdot L^{-1}$ in autumn and winter months, sometimes promoting intense water discoloration (Proença 2004). Lower abundances of *M. rubrum* in summer may thus have limited the occurrence of *Dinophysis* spp. inside Babitonga Bay during this study. In addition, there is limited evidence that *Dinophysis* populations may remain at low cell densities outside the estuaries along the adjacent continental shelf waters off the southern Brazilian coast (Haraguchi et al., 2015), even in summer months (Schmitt and Proença 2000; Villac et al. 2008). Therefore, it is possible that part of the high seasonal and interannual variability on *D.* cf. *acuminata* abundance reported herein can be explained by a mechanism of cell transport from marine waters into Babitonga Bay under certain atmospheric and oceanographic conditions, as suggested for other southern Brazilian coastal environments (Proença et al. 2007; Tibiriçá et al. 2015) and observed in other parts of the world such as Galicia in the Atlantic Spanish coast (Díaz et al. 2016) and Scotland (Whyte et al., 2014a).

In southern Brazil, periods of low precipitation coupled with constant winds from E to S quadrants, which become more frequent as winter approaches, lead to the transport of offshore waters towards the coast (Brandini et al., 2007), possibly bringing *Dinophysis* cells along, and favoring their retention inside the estuaries and coastal embayments. On a larger spatial scale, coastal water masses resulting from the encounter of the plumes of La Plata River and the Patos' Lagoon discharge at the Brazilian-Malvinas Confluence Zone (BMCZ), in the Southwestern Atlantic region, are predominantly transported northward during the autumn-winter season (Möller et al., 2008; Muelbert et al., 2008; Piola et al., 2008). Eventually, *Dinophysis* cells occurring in the southernmost Brazilian

continental shelf (Haraguchi and Odebrecht, 2010), as well as in Uruguayan (Martínez and Ortega, 2007) and northeastern Argentine coastal waters (Sar et al., 2012; Sunesen et al., 2014) may be carried along those water masses and reach the south-southeastern Brazilian coast during late autumn/early winter, as recently documented in 2016 (Proença et al. 2017).

Once inside the estuary, *Dinophysis* cells can multiply favored by factors such as increased water column stability (Reguera et al., 2012) and prey availability at sufficient cell densities, as reported for *D. acuminata* in the Galician Rías (Velo-Suárez et al. 2014) and Iberican Pensinsula (Moita et al. 2016). Other factors such as increased nitrogen and phosphorus inputs (Smayda 2008; Hattenrath-Lehmann et al., 2015), wind direction and velocity affecting the water column structure (Whyte et al., 2014b), and the synergistic interactions of decreased Redfield ratio, thermal stratification and variations in rainfall pattern (Ajani et al. 2016; Martínez et al. 2017) may also stimulate blooms of *Dinophysis* in other estuarine areas across the globe.

In the Southern Atlantic Ocean, the general atmospheric circulation pattern is characterized by convective movements often associated with low-pressure systems and intense rainfall during the warmest months (Bulgakov and Lomakin, 2002). During such conditions, estuaries and coastal environments experience high freshwater discharge triggering water column mixture. Heavy loads of nutrients are therefore made available by both lixiviation and re-suspension, which favors the dominance of diatoms within the phytoplankton assemblage (Procopiak et al., 2006; Díaz et al., 2013; Fernandes et al., 2013). In contrast, atmospheric high-pressure systems during cooler months usually provide more stable meteorological-oceanographic conditions. At this period, the abundance of diatoms tends to remain at low levels in the water column, as observed in other estuarine areas around the world (Cloern 1987; Ferreira et al., 2005; Villac et al. 2005; Artigas et al., 2014; Carstensen et al., 2015), leaving room for the co-dominance of populations of dinoflagellates such as *Alexandrium*, *Prorocentrum, Peridinium*, *Dinophysis* and others (Proença et al. 1999; Schmitt and Proença 2000; Omachi et al. 2007; Islabão and Odebrecht 2011; Haraguchi et al. 2015).

In the present study, peaks of *Dinophysis* abundance, as well as the occurrence of DSP episodes often associated with high OA concentrations in

mussels, were more intense during periods under the influence of La Niña (LNSO) than in other periods. In the southwestern Atlantic region, LNSO-dominated years (negative ENSO index) are characterized by cooler summers and drier winters than average, contributing to the formation of an anomalously colder Brazilian current and warmer Malvinas current (Severov, 2004). Both northward and shelf-to-coast transports, as well as the retention of *Dinophysis* cells inside estuaries, are expected to be intensified under these conditions, thus increasing the potential for DSP episodes during those years. In addition, there has been a recent increase in the intensity of *Dinophysis* blooms (higher cell density and duration) along the nearby Uruguayan coast, possibly associated with an increase in temperature and nutrient concentration, which has caused longer shellfish harvesting bans to be issued (up to 189 days in 2015) (Martínez et al., 2017).

During the investigated period, OA was detected in mussel tissues throughout the year, although more frequently and at higher levels from weeks 18 to 38 (mid-autumn to late winter), associated with more expressive cell abundances of *Dinophysis* spp. (mostly *D.* cf. *acuminata*). Although positive DSP-MBA results were more common during that same time of the year, some DSP events were registered in different periods and not always associated with the presence of significant OA amounts in mussels (e.g. 2014 episodes). This suggests that non-diarrheic lipophilic toxins (i.e., other than OA, DTX, and their derivatives) or other toxic compounds might be involved in some MBA-based shellfish harvesting shut downs, as reported in Jiang et al. (2014). Moderate to high levels of pectenotoxin-2 (PTX-2) and their derivatives (PTX2sa, 7-epi-PTX2sa) have been found in *D. caudata*, both in monoclonal cultures in Japan (Basti et al. 2015) and in single-cells sampled from the field in China (Li et al. 2015), and in *D. tripos* from North Patagonian gulfs associated to positive results in DSP-MBA (Villalobos et al., 2015). Although *D. caudata* was found in plankton net samples during the 2014 DSP outbreaks in Babitonga Bay, PTX-2 and derivatives were not detected by LC-MS/MS in any occasion in our study.

Conclusions

The data set presented herein indicates a reasonable risk for the occurrence of DSP outbreaks in this southern Brazilian estuary where low-

income families depend upon bivalve aquaculture as their source of income, arising concerns related to both public health and economic perspectives. The valuable information derived from this study can contribute to mitigate risks in this region mainly regarding the environmental conditions and periods more favourable to DSP occurrence (i.e. winter-spring; La Niña years), as well as the thresholds in *Dinophysis* abundance that trigger the initiation of both toxin accumulation and positive DSP-MBA results (~150 and 1200 cells L^{-1}, respectively). The DSP-MBA is still the main official regulatory method in use in Brazil. Despite being a valid and practical regulatory tool, false positive results generated by this method may compromise the relationship between stakeholders and shellfish growers, which can be decisive in the success of environmental and food safety monitoring programs (Draisci et al., 1994; Turrell and Stobo, 2007; Turner and Goya, 2016). Moreover, the persistence of diarrheic toxins in mussels during most part of the year, and across consecutive years, may add another health implication for frequent consumers subjected to chronical exposure to these substances (Vieira et al. 2013), which may potentially lead to gastrointestinal problems including increased tumor prevalence (Ito et al., 2008; Sosa et al., 2013).

Acknowledgement

We wish to express our gratitude to several shellfish farmers from Santa Catarina State for their help with sampling, to the State and National regulatory agencies (EPAGRI, CIDASC, and MAPA) for sharing monitoring data, and in special to the veterinarian Dr. Eduardo Cunha for his support and confidence that this study would contribute with relevant information to the Brazilian aquaculture.

CAPÍTULO 2 - Spatio-temporal distribution of *Mesodinium rubrum, Dinophysis* cf. *acuminata* and okadaic acid in a shallow subtropical estuary.

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Abstract

Harmful algal blooms related to toxin-producing species, such as *Dinophysis* spp., may cause serious economic and public health threats in shellfish-farming areas. This dinoflagellate has been reported in the southern Brazilian coast, responsible for 98% of the national shellfish aquaculture production. This study aims to determine the mesoscale-variation patterns in the horizontal and temporal distribution of *Dinophysis* spp., their prey (*Mesodinium rubrum*), and their toxins in a shallow and vertically homogeneous estuary. Depth-integrated water samples were collected from two regions (shallow and deep areas) near a major bivalve farming site. *D.* cf. *acuminata* (99% of the total abundance of the genus) and its prey occurred frequently, indicating that they are either well established within the estuary – even under unfavorable conditions – or transported northward by coastal currents and often brought into the estuary. The diarrheic toxin okadaic acid (OA) was detected both in the water particulate fraction and in the soft tissues of suspension-feeding bivalves (farmed mussels and wild clams), even when *Dinophysis* cell density was extremely low. Free-OA levels in mussels were closely associated with water turbidity, tidal amplitude, and wind speed, suggesting that part of the toxin incorporated could be adsorbed onto organic and/or inorganic particles in suspension and become available during turbulent periods. Whereas no biotic or abiotic parameter varied significantly between the two sampling areas, there was a marked seasonal variation in the abundance of *D.* cf. *acuminata* and its toxins, with higher levels attained from late autumn to spring. Considering the potential chronic effects of OA, the frequent contamination by low to moderate toxin levels (mean: 55 µg Kg^{-}

[1]) may thus represent a health risk to frequent consumers of bivalve mollusks in this region.

Key-words: DSP, Mussels, Clams, Shellfish Aquaculture, Harmful Algae.

Introduction

Harmful algal blooms (HABs) pose serious economic and public health threats in shellfish-farming areas, especially when they involve toxin-producing species (e.g., Morgan et al., 2009). The virulence, magnitude and spatial-temporal distribution of HABs vary according to the species or taxonomic group of the microalgae involved, mode of action of the toxic compound produced, type of organisms exposed to the toxin, and local meteorological and oceanographic conditions (Zingone and Enevoldsen, 2000; Smayda, 2008; Hallegraeff, 2010).

Negative effects from HABs are usually more remarkable in shellfish aquaculture areas. In Brazil, 98% of the 15,381 tons of bivalve mollusks produced per year come from the coast of Santa Catarina State (SC) in the southern coast (Santos and Costa, 2017). In that region, dinoflagellates producing paralytic toxins, such as *Gymnodinium catenatum* (Proença et al., 2001; Negri et al., 2007) and *Alexandrium* spp., (Omachi et al., 2007), as well as diarrheic toxins, such as *Prorocentrum lima* (Proença et al., 1999) and several species of the genus *Dinophysis* (Schmitt & Proença, 2000; Haraguchi & Odebrecht, 2010; Mafra et al., 2015b; Tibiriçá et al., 2015) have been reported. These occurrences are sometimes associated to deleterious effects such as those observed during blooms of *Dinophysis* cf. *acuminata* (Proença et al., 2007; Mafra et al., 2015a).

Lipophilic toxins causing the diarrheic shellfish poisoning (DSP) in humans upon consumption of contaminated seafood may represent one of the main obstacles to bivalve aquaculture in Brazil and elsewhere (review in Reguera et al., 2014). Accumulation of high levels of the toxin okadaic acid (OA) and its congeners – the *Dinophysis*toxins (DTXs) – in bivalve mollusks have been linked to the occurrence of *Dinophysis* spp. in the water, sometimes at cell densities as low as a few hundred cells per liter (Pavela-Vrančič et al., 2002; Swanson et al., 2010; Reguera et al. 2012; Mafra et al., 2014).

Toxigenic species of the genus *Dinophysis* are widely distributed in oceans and inner seas (Hallegraeff and Lucas, 1988), with most neritic populations occurring in sheltered coastal environments such as coves and estuaries

(Koukaras, 2004; Aissaoui et al., 2013). It is assumed that populations of *Dinophysis* spp. have their growth stimulated by specific environmental conditions (Rial et al., 2012) such as low water column turbulence (Díaz et al., 2013), moderate salinity (Díaz et al., 2011), availability of organic and inorganic sources of nutrients (Hansen et al., 2013), mostly nitrogen (Hattenrath-Lehmann and Gobler, 2015), as well as ecological aspects such as their trophic interaction with the co-occurring kleptoplastidic ciliate *Mesodinium rubrum* (e.g. Hansen, 2011; Mafra et al., 2016).

Much of the ecological knowledge about the genus *Dinophysis* comes from studies performed in deep coastal environments (e.g. fjords) associated with well-defined hydrodynamic patterns (Blanco et al., 2007; Alves-de-Souza et al., 2014). In such environments, the presence of this dinoflagellate is related to upwelling events, physical-chemical stratification of the water, and availability of prey (Hansen, 1991; Bravo et al., 1995; Velo-Suárez et al., 2014). In shallow and vertically homogeneous estuaries, however, the distribution and ecology of *Dinophysis* spp. remains poorly comprehended. In these later locations, the factors and processes affecting the abundance of *Dinophysis* spp. probably occur at shorter temporal and spatial scales. Thus, the present study aims to determine the mesoscale-variation patterns in the horizontal (hundreds of meters) and temporal (biweekly) distribution of these dinoflagellates, their prey, and their toxins in Babitonga Bay, a shallow estuary used as a bivalve mollusk farming area in southern Brazil.

Material and Methods

Study area

Babitonga Bay (Figure 1) is a shallow (average depth ~5 m), well-mixed estuary, dominated by semi-diurnal micro tides with uneven heights for consecutive low and high tide cycles (Truccolo and Schettini, 1999). It is located in a region characterized by humid subtropical climate, with prevailing winds from the northeastern quadrant, and average annual precipitation of 2,300 mm (Vieira et al., 2008). The aquaculture farming area of Paulas, where bivalve mollusks are

cultivated in suspended longlines (Silva, 2011), is located in the estuary's mouth next to an extensive sand-mud bank in the shape of a delta.

Sampling

Whole water-column samples (in triplicate) were collected bi-monthly during 13 months (from February of 2015 to February of 2016), using a hose (~2.5 L) deployed from a small boat, in two regions: the first at a shallow depth (S; ~2.0 m) and the second one at a deeper location (D; ~8.0 m), distant ~100 m from each other. In addition, concentrated samples were obtained through oblique trawls using a plankton net (20 □m mesh). Finally, in order to determine the content of diarrheic toxins in soft tissues of bivalves, samples of *Anomalocardia brasiliana* clams (n = 10 individuals) were collected from a natural bank located in the S region, and those of *Perna perna* mussels (n = 20 ind.) were collected from suspended longlines, seated between regions S and D.

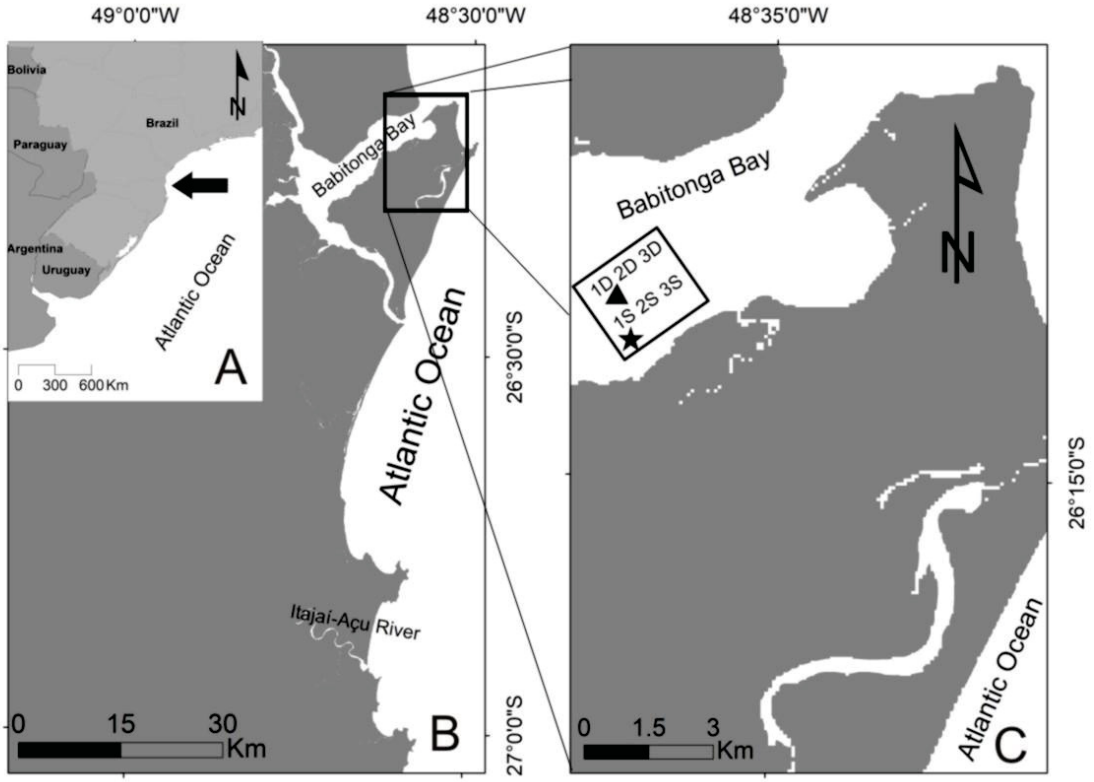

Figura 6 - Cap 2: Figure 1 – Study area, Babitonga Bay, showing (A) its geographical location along the southern Brazilian coast; (B) general view of the northern shore of Santa Catarina State; and (C) detailed view of its outer portion, featuring the aquaculture farming area of Paulas (solid rectangle), where the sample sites for phytoplankton (n=3) were distributed along a deep ("D") and a shallow ("S") region, and the collection sites for mussels (black triangle), and clams (black star) were located.

Measurements of air temperature (°C) and wind speed (m s^{-1}) were taken *in situ* during the sampling campaigns, using a Kestrel 400 portable weather station. Water temperature and salinity were measured at every 0.5 m, from the surface down to 4.5 m deep, using a YSI Professional plus multi-parameter probe. Water turbidity was measured with a TECNOPON portable turbidimeter, and the water transparency estimated with a Secchi disk. Finally, tidal amplitude was calculated from the information on daily maximum and minimum heights provided by the Brazilian Navy to the nearby port of São Francisco do Sul (BRASIL, 2016).

Sample processing

Plankton-net samples were preserved in 4% formaldehyde solution and destined for microphytoplankton identification by morphometric analysis. In addition, one 300-mL aliquot of each water sample was preserved with 1% Lugol solution for the quantitative phytoplankton analysis, and two extra 300-mL aliquots were gently vacuum-filtered through glass-fiber filters (47 mm diameter, 0.45 µm porosity; Marcherey-Nagel® 85/70BF). One filter was used for the analysis of photosynthetic pigments and the second one for the determination of diarrheic toxins in the suspended particulate matter (SPM). Part of the filtered volume (200 mL) was collected and maintained frozen for the determination of dissolved macronutrients.

Filters were later soaked in 4 mL of HPLC grade methanol (99.5%) and exposed to an ultrasonic probe (Cole Parmer, CPX130) for 30 s at 80% of the total amplitude (130 W) for disruption of the retained cells, with the samples immersed in ice bath during the process. Extracts were filtered through syringe filters (PVDF, 13 mm in diameter and 0.22 µm porosity; Analitica®) to remove the remaining debris, and then collected into centrifuge microtubes (1.5 mL), remaining frozen in the dark until the analyses for the determination of photosynthetic pigments and diarrheic toxins, as described later.

Bivalve mollusks were removed from their shells and their flesh mashed with an IKA UltraTurrax homogenizer. Methanol (99.5%, HPLC grade) was added

in the ratio of 1:9 (v:v) to 1.0 ± 0.5 g of homogenate, mixed in a vortex mixer for 3 min and centrifuged at 2000 × g for 10 min. The supernatant was filtered directly into a 2.0 mL glass vial through a syringe filter and kept frozen until the analysis of diarrheic toxins in their free forms. Subsequently, one 1-mL aliquot of the extract was subjected to alkaline hydrolysis mediated by the addition of 2.5 M sodium hydroxide in a 76 °C thermal bath for 40 min to convert the conjugated (metabolized) forms of toxins into their free forms, followed by the addition of 2.5 M hydrochloric acid for pH neutralization. The amount of conjugated toxins was obtained by discounting the concentration of free toxins initially measured in the non-hydrolyzed extract from the total concentration of toxins obtained from the hydrolyzed extract.

Quantification of phytoplankton

Counting of *Dinophysis* and *M. rubrum* cells was performed in a 50-mL aliquot of the Lugol-fixed sample. These samples were concentrated down to 10 mL (5×) by reverse filtration through a 10 µm porosity nylon screen, and allowed to settle for 24 h in Utermöhl chambers (Edler and Elbrächter, 2010). Cell counting was performed by scanning the whole chamber under an inverted optical microscope at 200 x magnification (limit of detection, LOD: 20 cells L^{-1}). The main microalgal groups (total cryptophytes, total dinoflagellates, total diatoms, and total microphytoplankton) were quantified in volumes ranging from 10 to 20 mL (depending on the sample turbidity) by counting cells in 5-10 random microscope fields of view (LOD: 50-100 cel L^{-1}), after 24-h settlement.

Spectrophotometric analysis of the dissolved inorganic nutrients

Aliquots (25 mL) of the filtered seawater were used to determine the concentrations of phosphate ($P\text{-}PO_4^{3-}$), nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$), ammonium ($N\text{-}NH_4^+$), and silicate SiO_2^-) by means of colorimetric methods (Grasshoff et al., 1999). The concentrations were determined from a linear regression obtained from successive dilutions of the respective analytical standards, with adjustment higher than 90% ($r^2 > 0.90$).

Analysis of photosynthetic pigments by liquid chromatography coupled to diode array detection (LC-DAD)

The methanolic extracts were injected (100 µL) into a liquid chromatography system (Hitachi® Chromaster) composed of a quaternary gradient pump, an automatic thermostat injector, a column oven (set at 40 °C), and a photodiode detector (DAD). Samples were eluted in a mixture of (A) methanol:acetone:pyridine (50:25:25) and (B) acetonitrile:acetone (80:20), at 1.0 mL min^{-1}. The proportion of B increased from 0 to 40% during 18 min, and then to 100% within the following 4 min of analysis, remaining at 100% for an extra 16-

min period and returning to the initial conditions (0% B) after 2 min (40 min in total). The pigments were confirmed by analyzing their retention times after the chromatographic separation in a Waters Symmetry® C8 column (150×4.6 mm, 3.5 µm particles), confronted to their typical absorbance spectra (350-750 nm scan), according to the methodology described by Zapata et al. (2000). The chlorophyll-*a* concentration was calculated by means of linear regression obtained from successive dilutions of the analytical standard (Sigma-Aldrich) (0.8, 1.6, 3.1, 6.2, 12.5, and 25 ng mL^{-1}) with trend adjustment higher than 98% ($r^2 > 0.98$). The relative abundances of accessory pigments were calculated as ratios of the peak area for each pigment in relation to that of chlorophyll-*a* in a given sample.

Analysis of diarrheic toxins by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

The detection of diarrheic toxins occurred in a liquid chromatography system (Agilent Technologies® 1260) coupled to a triple quadrupole mass spectrometer (AB Sciex® qTRAP 3200) equipped with a turbo ion spray (ESI) ionization source, following the official analytical method harmonized by the European Union (EURLMB, 2015). Five to 15 µL of each sample were injected and eluted at 0.3 $mL.min^{-1}$ by the mobile phase consisting of a mixture of (A) 100% ultra-pure water and (B) 95% acetonitrile, both with the addition of ammonium formate (2 mM) and formic acid (50 mM). The initial fractionation rate of 80:20% (A:B) increased to 100% B during the first 8 min of analysis, thus remaining for 3.5 min, and returning to the initial condition until the end of the analysis (13 min). Compounds were separated on a C18 column (Agilent Poroshell®, 50×2.1 mm, 2.7 µm particles), maintained at 20 °C. The identification of each toxin was achieved from their retention times and the mass spectra of the transition ions present in the samples in relation to the same parameters obtained for the analytical standards. High-purity air heated to 500 °C was used as the nebulizing gas. The other general conditions of the MS/MS detection system and the transition ions scanned for each investigated toxin are presented in Table 1.

Table 3 - Cap 2: Table 1: Mass spectrometry (MS/MS) conditions in negative electron spray (ESI) ionization mode. Q1: quadrupolo 1, Q3: quadrupolo 3, DP: declustering potential, EP: entrance potential, CEP: collision cell entrance potential, CE: collision energy and CXP: collision cell exit potential selected for each transition ion investigated during the toxin analysis.

Toxin	Q1 (m/z)	Q3 (m/z)	DP (v)	EP (v)	CEP (v)	CE (v)	CXP (v)
OA	803.5	255.0	-129	-10	-40.1	-82	-2
OA	803.5	113.0	-129	-10	-41.5	-64	-2
DTX-2	803.5	255.0	-129	-10	-40.6	-64	-2
DTX-2	803.5	113.0	-129	-10	-41.5	-84	-2
DTX-1	817.5	255.0	-129	-10	-41.5	-62	-2
DTX-1	817.5	113.0	-120	-10	-51.7	-82	-2

The quantification was carried out by means of calibration with an external standard, from a calibration curve built with certified reference material (IMB-NRC, Canada) dissolved in methanol for OA, and in matrix composed of mussel tissues (CRM-DSP-Mus-b) for DTX-1. Quantification of OA was based on the equation obtained from the linear regression (r^2> 0.95) of the following concentrations: 3.5, 14, 56, and 223 ng mL^{-1}.

Data analysis

Data were treated and analyzed critically for consistency, and then subjected to statistical tests with the aid of R studio software (R Core Team, 2017). Kruskal-Wallis non-parametric test, followed by the Dunn test, was applied to comparative analyses within and between treatments (regions D and S) and to the analysis of variation over time, using the dunn.test package (Dinno, 2017). In order to quantify the degree of relationship between the biotic and abiotic parameters, a multivariate principal components analysis (PCA) was applied for the best view of variable associations, using the FactoMiner package (Lê et al., 2008).

Results

Abiotic parameters

The weekly-averaged air temperature varied at a greater degree during fall (April-June; 10 ºC amplitude) compared to spring (September-December). The average wind speed during the entire sampling period was 1.0 m s^{-1}, although gusts of up to 7 m s^{-1} were recorded in some sampling campaigns, especially in late spring (November-December). The mean tidal amplitude was 1.3 m, with maximum peaks of 1.9 m during spring tides and minimum of 0.5 m in neap tides (Fig. 2C).

Water temperatures (Fig. 2B) reached a minimum of 19.2 °C in July (winter) and a maximum of 27.7 °C in February (summer). Salinity values were higher (above 27 PSU) from June to September, the drier period, contrasting with much lower values (up to 6-7 PSU lower) measured during the following months (November-April), the warmer and more rainy period of the year (Fig. 2B); values increased significantly ($H = 89.3$; $p = 0.01$) again from April to June. Transparency, as estimated by the Secchi disk depth, averaged 1.0 m throughout the study period (Fig. 2C); lower values were registered in the shallower area, mainly because the disk touched the bottom and the values were thus underestimated during periods of increased water transparency (June and January). The lowest values of water transparency (0.6 m) and the highest values of turbidity (21 NTU) were observed from September to December (spring). Turbidity values were similarly high in both shallow and deep areas, indicating a high load of SPM (organic and inorganic) in the bay.

Concentrations of dissolved inorganic nutrients (DINs) were slightly higher in the deeper site relative to the shallower area, with a general increase registered from September to November, especially for SiO_2^-, PO_4^{3-}, and NH_4^+ (Fig. 3A-B). Nitrogen was predominant in the reduced form (NH_4^+; mean = 3.3 µM); concentrations of oxidized forms varied much less in time and space, remaining at moderate to low levels for both NO_3^- and NO_2^- (mean = 1.9 and 1.2 µM, respectively). Phosphate concentrations were always below 6.3 µM, whereas

SiO_2^- was the most abundant macronutrient available in the estuary (min: 11.7; max: 102.8 µM), especially from September to December (Fig. 3A-B).

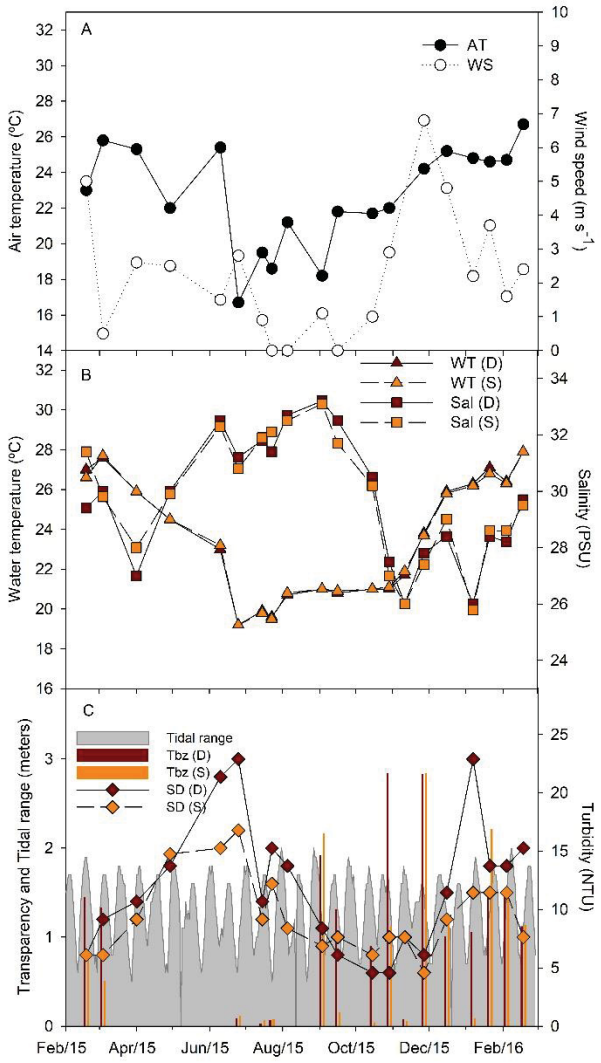

Figura 7 - Cap 2: Figure 2 – Meteorological and water physico-chemical parameters measured in situ in the deep (D: deep) and shallow (S: shallow) areas: (A) absolute values of air temperature (AT) and wind speed (WV) during sampling campaigns; (B) mean values along the water column for water temperature (WT) and salinity (Sal); and (C) mean water turbidity values (Tbz) and absolute values of tidal amplitude (shadowed area) and water transparency, as estimated by the Secchi disk depth (SD).

Composition and distribution of phytoplankton

The concentration of chlorophyll-*a*, a proxy for total phytoplankton biomass, averaged 0.54 mg m^{-3} (maximum of 2.6 mg m^{-3}) throughout the study, with the highest concentrations measured during the hottest months (February and March) and in the shallower region (Fig. 3C-D). Diatoms were consistently dominant over the study period; their abundance decreased significantly from

August to October (as did the whole phytoplankton community), but values increased back after December (Fig. 3C-D). The profiles of photosynthetic pigments (Fig. 3E-F) were consistent with the composition of the microphytoplankton community. Fucoxanthin was the most frequent and abundant pigment in proportion to chlorophyll-*a*, followed by chlorophyll-*b*, c_2, and c_3 in both sampling areas (Fig. 3E-F). In addition, there was a constant detection of moderate levels of dinoxanthin and diadinoxanthin.

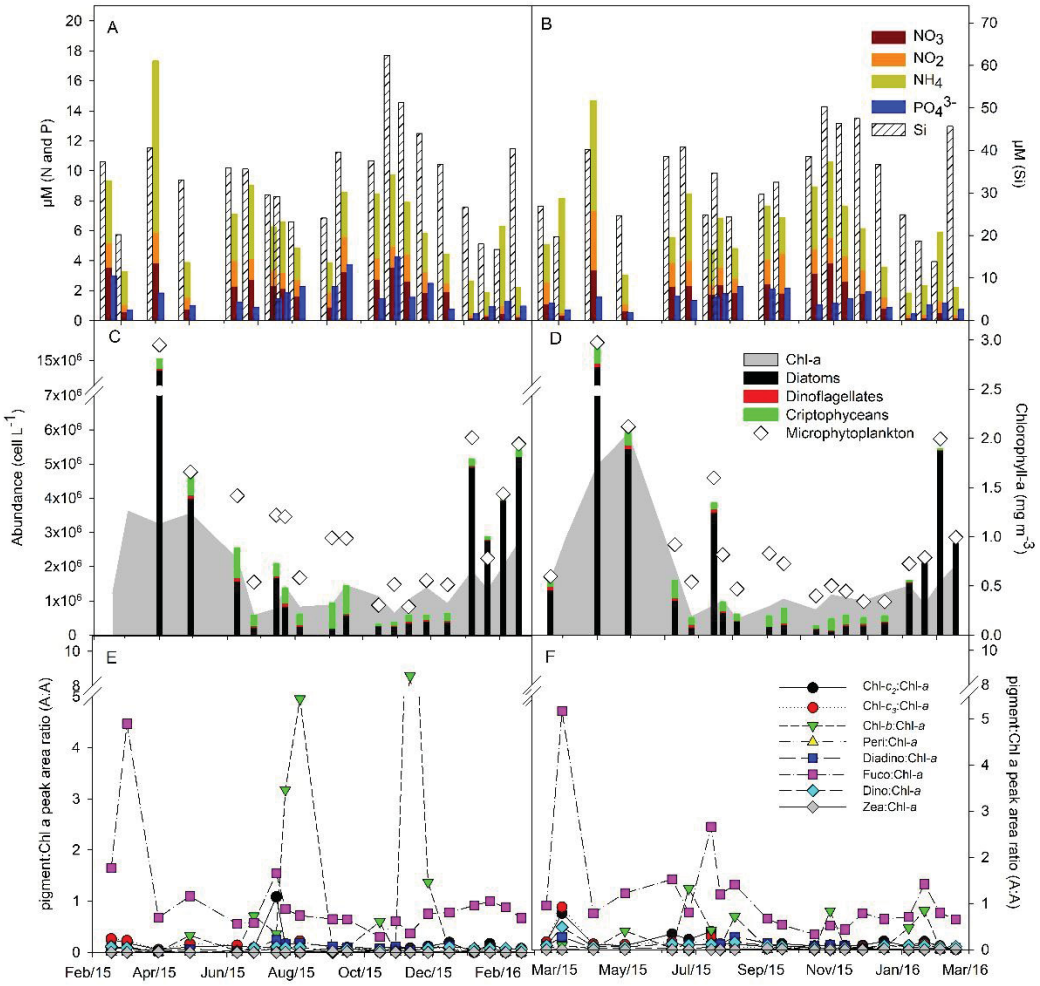

Figura 8 - Cap 2: Figure 3 – Mean concentration values of (A-B) dissolved inorganic nutrients, (C-D) cell density of the main taxonomic groups and of the total microphytoplankton, as well as chlorophyll-a concentrations, and (E-F) ratios of the following pigments to chlorophyll-a in the (E) deep and (F) shallow sampling areas: chl-c3: chlorophyll-c3, chl-c2: chlorophyll-c2, chl-b: chlorophyll-b, Peri: peridin, Diadino: diadinoxanthin, Fuco: fucoxanthin, Dino: dinoxanthin, and Zea: zeaxanthin.

The ciliate *M. rubrum* occurred frequently at low cell abundances throughout the investigation period, attaining a maximum of 600 to 800 cells L^{-1} during winter and spring (July to November), with no significant variation between sampling areas (Figure 4A). *D.* cf. *acuminata* reached the highest cell

abundances (up to 120 ± 20 cells L^{-1}) during late autumn and winter (late May to August), with a second abundance peak (70 ± 20 cells L^{-1}) coinciding with the period of highest density of its prey, *M. rubrum*, in spring (October), and a third peak (40 ± 20 cells L^{-1}) during the austral summer (Figure 4C). *Dinophysis caudata, Dinophysis tripos, D. scrobiculata, D. sacculus and P. rotundatum* (Appendix 1) were occasionally detected on plankton net samples, mainly from July to October.

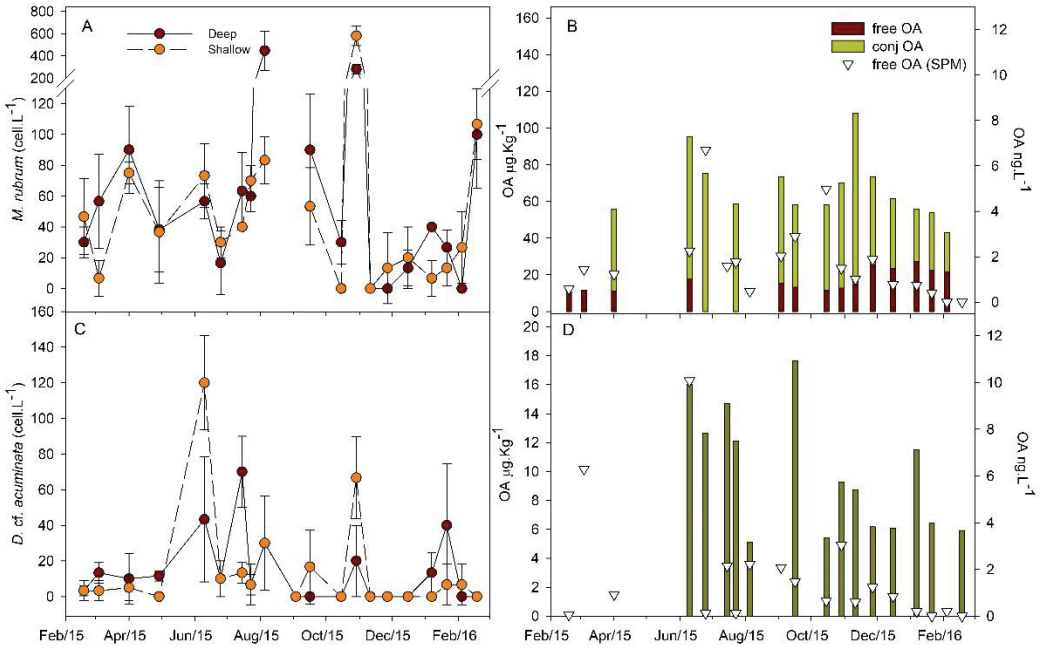

Figura 9 - Cap 2: Figure 4 – Mean cell density (± standard deviation; n = 3) of (A) Mesodinium rubrum and (C) Dinophysis cf. acuminata in the deep and shallow sampling areas; and mean concentration of okadaic acid accumulated by (B) Perna perna mussels and (D) Anomalocardia brasiliana clams in both free (free OA) and conjugated (conj OA) forms, expressed in µg Kg^{-1}, as well as free OA in suspended particulate matter (SPM; inverted white triangles), expressed in ng L^{-1}, in the (B) deep and (D) shallow sampling areas.

Diarrheic toxins

Okadaic acid (OA) was the only diarrheic toxin found both in the suspended material and in bivalve mollusks, mainly from winter to summer (Fig. 4B, D), associated with the presence of *D.* cf. *acuminata* in the water. In the particulate fraction, the highest toxin amounts (up to 10.0 ng L^{-1}) were detected in late fall/ early winter, with a second peak (~5 ng L^{-1}) during spring.

The highest OA amounts accumulated in bivalves (max. ~100 µg Kg^{-1}) were detected in mussels (Fig. 4B), which were suspended in longlines between the deep and the shallow areas. The toxin in its free (i.e., non-metabolized) form

was relatively more abundant at the end of spring and in summer (41% of the total OA, on average, during such period), occurring more expressively after the second peak of *D.* cf. *acuminata* abundance in spring. The conjugated forms of OA were dominant in mussels (77% of the total OA, on average), especially in winter and spring when toxin concentrations were significantly higher ($H = 71.1$; $p = 0.01$). In addition, OA was only found in its conjugated forms in clams, harvested directly from muddy-sandy banks in the inter-tidal region, with levels constantly below 20 μg Kg^{-1} from late fall to summer (maximum values measured in winter) (Fig. 4D).

Correlations

Free OA levels in mussels were directly associated with water turbidity and wind velocity, as assessed by the multivariate analysis of principal components, which explained 35.7% of data variability (Fig. 5). Moreover, there was an association of total OA levels in both mussels and clams with salinity, water transparency and the concentrations of nitrate and nitrite. This grouping was also associated, although to a lesser extent, with the availability of free OA in the seston (SPM) and the abundance of *D.* cf. *acuminata* cells in the water column (Fig. 5). In contrast, the abundance of diatoms – the main phytoplankton taxonomic group in this estuary and thus related to the abundance of total phytoplankton and the concentration of chlorophyll-*a* – was also associated with the reduced nitrogen form, ammoniu*M.*

Discussion

Both the toxigenic dinoflagellate *D.* cf. *acuminata* (99% of the total abundance among all *Dinophysis* spp.) and its prey, the ciliate *M. rubrum,* occurred frequently – although at low cell abundances – during the investigated period in Babitonga Bay. Even though, measurable and sometimes relatively high amounts of okadaic acid were detected in the water particulate fraction (seston), as well as in the soft tissues of suspension-feeding bivalves throughout the sampling period. More importantly, the present study indicates that the accumulation of this toxin may occur in both farmed mussels, suspended in the

water column, and in natural, intertidal populations of clams, even under conditions of extremely low cell densities of these dinoflagellates in the estuary. In general, there were no significant differences between the two sampling areas (deep and shallow) in terms of values and seasonal variation patterns of the biotic and abiotic parameters measured. The small distance between the sites (~250 m) and the intense hydrodynamics of this tide-dominated estuary are probably the factors responsible for such homogeneity, possibly indicating that our data is representative of a larger area than the sampling zone.

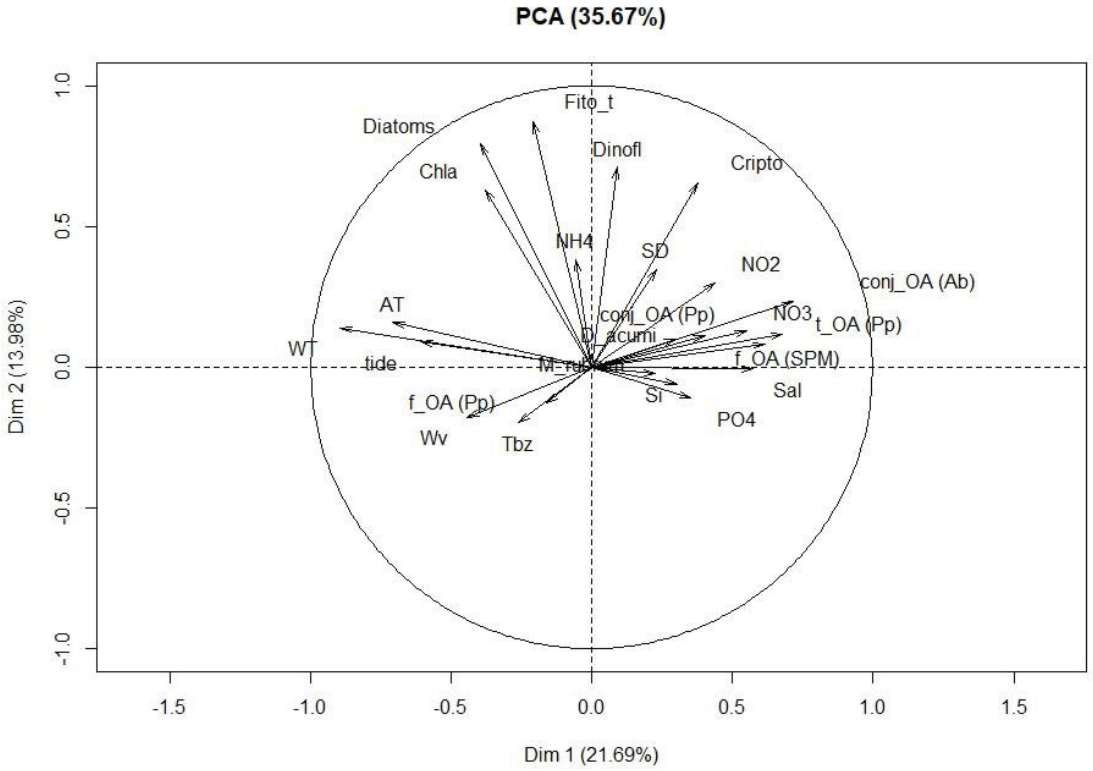

Figura 10 - Cap 2: Figure 5 – Principal components analysis explaining 35.67% of the data variability, decomposed in first axis (Dim 1: 21.69%) and second axis (Dim 2: 13.98%), for the following abiotic and biotic factors: water temperature (WT), air temperature (AT), tidal range (tide), wind velocity (Wv), salinity (Sal), Secchi disk depth (SD), turbidity (Tbz), cell density of Dinophysis cf. acuminata (D_acumi), Mesodinium rubrum (M_rubrum), criptophyceans (Cripto), diatoms (Diatoms), dinoflagellates (Dinofl) and total microphytoplankton (Fito_t), concentration of chlorophyll-a (Chla), phosphate (PO4), nitrate (NO3), nitrite (NO2), ammonium (NH4), silicate (Si), free okadaic acid (OA) in Perna perna (f_OA (Pp)), conjugated OA in P. perna (conj_OA (Pp)), total OA in P. perna (t_OA (Pp)), conjugated OA in Anomalocardia brasiliana (conj_OA (Ab)) and free OA in suspended particulate matter (f_OA (SPM)).

During the investigated period, the abiotic parameters reflected the typical seasonal variation pattern expected for subtropical coastal environments in Brazil (Martins et al., 2014). As previous studies in adjacent areas (Fernandes et al., 2002; Fujita and Odebrecht, 2007; Tibiriçá et al., 2015), both air and water

temperatures were higher during the austral summer (December 21st to March 20th) and salinity values were greater during cooler and drier months (late fall to late winter) in Babitonga Bay. The influence of marine waters in the outer portion of the estuary, as assessed by higher salinity and transparency values, was more remarkable in winter (June to August), coinciding with a period of reduced wind velocities (i.e. increased meteorological stability). In contrast, from early spring to late summer (September to March), stronger winds were accompanied by an increase in turbidity and the concentration of nutrients such as SiO_2^-, NH_4^+, and PO_4^{3-} inside the estuary. In fact, the availability of DINs was directly related to both turbidity and wind speed, ultimately affecting the abundance of the investigated phytoplankton taxonomic groups.

Chlorophyll-*a* was directly correlated with the abundance of diatoms, demonstrating the important contribution of this taxonomic group for the primary production in this portion of the estuary. Moderate concentrations of this pigment were measured during the period of higher temperature, wind speed and turbidity. The elevated relative abundance of fucoxanthin during periods of high chlorophyll-*a* concentration corroborates the dominance of diatoms within the phytoplankton assembly, which is consistent with previous results reported in this environment (Parizzi et al., 2016) and in other estuaries across the globe (Moreno et al., 2012; Madhu et al., 2014; Carstensen et al., 2015). In addition, based on unique combinations of chlorophylls and carotenoids , the usual co-occurrence of other pigments such as peridinin, dinoxanthin, diadinoxanthin and the chlorophylls c_2, c_3 and *b* might reflect the frequent presence of distinct dinoflagellates of different chloroplast types (Zapata et al., 2012) and other phytoplankton and phytobenthic groups, such as Haptophytes, Chlorophyceans and Prasinophyceans (Carreto *et al.*, 2003; Brotas; Plante-Cuny, 2003).

The inverse relationship between silicate concentration and diatom abundance, and the direct relationship between nitrogen and dinoflagellates (e.g., *Dinophysis* spp.), as registered in the present study, have been previously observed in subtropical estuaries from different regions, such as Spain (Martínez-Guijarro et al., 2013) and Turkey (Polat, 2002). Nonetheless, it should be noted that high hydrodynamics as well as relatively intense anthropic perturbations such as dredging and maritime traffic (Silveira et al., 2012); may contribute to

remobilize and suspend nutrients and others compounds from the bottom (Martins et al., 2014; Rizzi et al., 2017), making it difficult to establish a relationship between the concentration of nutrients and the dominance of specific taxonomic groups in the outer portion of Babitonga Bay.

The occurrence of *M. rubrum* in our study was not directly associated with the presence of cryptophycean microalgae, which could indicate the ability of these organisms to retain the sequestered chloroplasts functionally active for extended periods, as observed in laboratory experiments (e.g. Myung et al., 2013). Alternatively, although only cryptophytes can sustain high growth rates in *M. rubrum* (Hansen et al., 2012), the ciliate may sometimes ingest other protistan and prokaryote prey, such as heterotrophic nanoflagellates and bacteria (Seong et al., 2017), as well as the autotrophic cyanobacterium *Synechococcus* (Yoo et al., 2015), which may be eventually available in Babitonga Bay as suggested by the presence of trace amounts of zeaxanthin and trace amounts of several other carotenoids and phycobiliproteins in the water (Kana et al., 1988; Xia et al., 2018). In any case, the frequent occurrence of the kleptoplastidic ciliate *M. rubrum* throughout the year denotes its resident character within the estuary. Our results thus demonstrate that, like in Chesapeake Bay, USA (Johnson et al., 2013), populations of this ciliate tolerate broad environmental variability, occurring at greater abundance under conditions of low salinity and water temperature, ultimately allowing *Dinophysis* populations to develop over a prolonged period.

Hansen (1991) first provided reliable evidence that some *Dinophysis* species can act either as a prey or predator of ciliates. Although recent studies have indicated that *Dinophysis* spp. may eventually prey upon and acquire their plastids (i.e., kleptoplasty) from ciliates other than *M. rubrum* (Nishitani et al., 2012; Kim et al., 2015), this species is by far the main prey item sustaining *Dinophysis* spp. growth worldwide (reviewed in Reguera et al., 2012). In our study, increases in the abundance of *M. rubrum* were accompanied by the presence of *D.* cf. *acuminata* cells in the water, supporting the natural co-occurrence/succession of these organisms, as previously documented in this (Parizzi et al., 2016) and other regions (Sjöqvist and Lindholm, 2011; Kim et al., 2012; Stern et al., 2014).

The frequent occurrence of *D.* cf. *acuminata* in this portion of the estuary suggests that its populations are either well established within the estuary even under unfavorable conditions, as reported by Johnson et al. (2013), or are often brought into the estuary by coastal currents and rising tides. In fact, populations of *Dinophysis* spp. that are commonly found further south along the southern Brazilian continental shelf (Haraguchi & Odebrecht, 2010), as well as in Uruguay (Martínez & Ortega, 2007; Martínez et al., 2017) and Argentina (Sar et al., 2012; Sunesen et al., 2014), may be transported northwards together with the displacement of the Plata Plume Water (PPW) during winter (Möller et al., 2008; Muelbert et al., 2008; Piola et al., 2008), sometimes reaching Babitonga Bay area or even lower latitudes. The question whether the inoculum for recurrent *D.* cf. *acuminata* blooms (see Chapter 1, this thesis) stays inside the estuary or is transported inward, or both, remains to be properly addresse*D.*

The presence of *D.* cf. *acuminata* in the outer portion of the estuary, next to the shellfish farming area, was directly related to the levels of particulate OA measured in the water. The total OA in both bivalves and associated with salinity and nitrogen dissolved might indicate greater shellfish contamination under periods of marine influence inside the estuary. Conversely, the amount of OA accumulated in its free form by farmed mussels (*P. perna*) exhibited no correlation with the cell density of *D.* cf. *acuminata*, or of any other phytoplankton taxonomic group. Instead, free-OA levels in mussels were very significantly and directly related to water turbidity, tidal amplitude, and wind speed. This suggests that at least part of the toxin incorporated by the mussels could be initially adsorbed onto organic and/or inorganic particles in suspension (Munday and Reeve, 2013; McCarthy et al., 2014), and that toxin uptake by suspension-feeding bivalves could be thus favored by the remobilization and further transport of toxin-coated particles in the water column. Therefore, considering the great amounts of diarrheic toxins usually released into the water by *Dinophysis* cells during laboratory experiments (e.g. Mafra et al., 2016), the adsorbed toxin fraction may become more relevant to the management point of view than *Dinophysis* cell density or the intracellular toxin quota itself.

Mussels (*P. perna*) exhibited 3 to 10-fold higher OA levels than clams (*Anomalocardia brasiliana*) during the present study, which might reflect different

exposure degrees to the toxin according to their habitats (Vale, 2006). Benthic populations of clams feed on particulate material such as sediment, organic matter, toxic algae and algal-derived detritus, whose availability is mainly controlled by the estuary circulation and particle sinking rates (Reizopoulou et al., 2008). Additionally, the relationship between water turbidity and the OA amounts accumulated by the endopsamic bivalve *A. brasiliana*, as detected in Babitonga Bay, may suggest a role of secondary trophic pathways through the ingestion of particulate organic material, alive or not (Villar-González et al., 2007; Vale et al., 2008; Armi et al., 2012; Sar et al., 2012), in toxin uptake by this species. Finally, interspecific differences in toxin accumulation, as commonly reported for other bivalve species and toxins (Bricelj and Shumway, 1998; Reizopoulou et al., 2008; Mafra et al., 2010; 2015b), can be also attibuted to differential uptake, body size, tissue allocation (e.g. (Mafra et al., 2010a) and/or differential metabolism/degradation during the detoxification process. For diarrheic toxins, detoxification is mainly mediated by enzymatic processes that make the toxin more water soluble via conjugation with endogenous molecules (MacKenzie et al., 2012), facilitating its elimination through the metabolic pathway of xenobiotics (Svensson, 2003; Franco et al., 2006; Mello et al., 2010; Nielsen et al., 2016). In the present study, most of the toxin burden accumulated by farmed mussels and all OA present in wild clams was already in the conjugated (i.e. metabolized) form, indicating that both bivalve species possess a rapid/efficient detoxification mechanis*M.* Free-OA absence in clams (from intertidal sandy banks) might be a pelagic-benthic coupling evidence (Sohma et al., 2008), in which the OA uptake occurs through some secondary pathway as zooplankton, faecal pellets or detritus (particles falls) ingest (Maneiro et al., 2002a; Turner, 2014). The same OA form ratio pattern were observed between Danish surf clams (*Spisola* spp.) and blue mussels (*Mytilus edulis*) on Denmark (Jørgensen et al., 2005). Even though, slight differences in toxin transformation rates, as reported by Vale (2004) for blue mussels (*Mytilus galloprovincialis*), common cockles (*Cerastoderma edule*), razor clams (*Solen marginatus*) and clams (*Ruditapes decussata, Venerupis pullastra*), could at least partly explain the interspecific differences in OA accumulation reported herein.

The amount of total (i.e. free + conjugated) OA accumulated in bivalve tissues was greater after periods of higher *Dinophysis* cell abundance in the

estuary (July–September). However, toxin levels remained always below the regulatory limit for DSP (160 µg Kg^{-1}). Nevertheless, in a recent review on the toxic effects of OA and its congeners, Valdiglesias et al. (2013) concluded that the minimum level affecting humans (LOAEL – *lowest observed adverse effect level*) is 50 µg of OA equivalent per person, on average. This suggests that mild cases of food poisoning may have occurred among consumers of large portions (i.e. ~500 g) of contaminated bivalves in the studied region. Moreover, besides acute gastrointestinal effects, it has been suggested that OA and other lipophilic toxins can cause molecular, cellular, and genetic expression alterations and even promote the appearance and development of tumors upon chronic exposure (Aune et al., 2007; 2012; Ito et al., 2008; Sosa et al., 2013; FAO/WHO, 2016). The consumption of bivalve mollusks from Babitonga Bay, which were frequently contaminated with low to moderate levels of OA in this study (mean of 55 µg Kg^{-1}, maximum of 107 µg Kg^{-1}), may thus represent a health risk to frequent consumers of these organisms.

Acknowledgments

The authors are thankful to the bivalve mollusk producers linked to the Association of Marine Farmers of Paulas (AMAP) and to the personnel involved in HAB monitoring programs in Santa Catarina State, including those from Agricultural Research and Rural Extension Company (EPAGRI), Integrated Company of Agricultural Development (CIDASC), of Santa Catarina and the Agricultural and Livestock Defense Secretary (SDA) of the Ministry of Agriculture, Livestock and Food Supply (MAPA).

CAPÍTULO 3 - Diel Variations in Cell Abundance and Trophic Transfer of Diarrheic Toxins during a Massive *Dinophysis* Bloom in Southern Brazil

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Abstract

Dinophysis spp. are a major source of diarrheic toxins to marine food webs, especially during blooms. This study documented the occurrence, in late May 2016, of a massive toxic bloom of the *Dinophysis acuminata* complex along the southern coast of Brazil, associated with an episode of marked salinity stratification. The study tracked the daily vertical distribution of *Dinophysis* spp. cells and their ciliate prey, *Mesodinium* cf. *rubrum*, and quantified the amount of lipophilic toxins present in seston and accumulated by various marine organisms in the food web. The abundance of the *D. acuminata* complex reached 43 × 104 cells·L−1 at 1.0 m depth at the peak of the bloo*M.* Maximum cell densities of cryptophyceans and *M.* cf. *rubrum* (>500 × 104 and 18 × 104 cell·L−1, respectively) were recorded on the first day of sampling, one week before the peak in abundance of the *D. acuminata* complex. The diarrheic toxin okadaic acid (OA) was the only toxin detected during the bloom, attaining unprecedented, high concentrations of up to 829 µg·L−1 in seston, and 143 ± 93 pg·cell−1 in individually picked cells of *the D. acuminata* complex. Suspension-feeders such as the mussel, *Perna perna*, and barnacle, *Megabalanus tintinnabulum*, accumulated maximum OA levels (up to 578.4 and 21.9 µg total OA·Kg−1, respectively) during early bloom stages, whereas predators and detritivores such as Caprellidae amphipods (154.6 µg·Kg−1), *Stramonita haemastoma* gastropods (111.6 µg·Kg−1), *Pilumnus spinosissimus* crabs (33.4 µg·Kg−1) and a commercially important species of shrimp, *Xiphopenaeus kroyeri* (7.2 µg·Kg−1), only incorporated OA from mid- to late bloom stages. Conjugated forms of OA were dominant (>70%) in most organisms, except in blenny fish, *Hypleurochilus fissicornis*, and polychaetes, *Pseudonereis palpata* (up to 59.3 and 164.6 µg total

OA·Kg−1, respectively), which contained mostly free-OA throughout the bloo*M.* Although algal toxins are only regulated in bivalves during toxic blooms in most countries, including Brazil, this study indicates that human seafood consumers might be exposed to moderate toxin levels from a variety of other vectors during intense toxic outbreaks.

Keywords: HAB; Okadaic acid; Field-study; Marine biota; Small-scale

Introduction

The frequency, duration and severity of *Dinophysis* blooms have increased worldwide over the past two decades (Reguera et al., 2012), leading to numerous episodes of massive shellfish contamination by lipophilic toxins in Europe (García-Altares et al., 2016; Whyte et al., 2014b), Africa (Aissaoui et al., 2014; Pitcher et al., 2011), Asia (Li et al., 2015), North America (Hattenrath-Lehmann et al., 2013), and South America (Martínez et al., 2017; Sar et al., 2012; Villalobos et al., 2015). Although scientific evidence indicates that the increase in harmful algal blooms may be correlated with meso- and large-scale physico-chemical processes, i.e., artificial eutrophication (Buskey, 2008; Heisler et al., 2008; Lewitus et al., 2008; Smayda, 2008), and global climate change (Hallegraeff, 2010; O'Neil et al., 2012), possible causes for an apparent increase in *Dinophysis* blooms are less comprehended.

Neritic and oceanic *Dinophysis* spp. are frequently observed in offshore waters along the southern coast of Brazil (Haraguchi and Odebrecht, 2010). In 2007, the first large-scale bloom of *Dinophysis* cf. *acuminata* complex ever reported in this region caused intoxication of at least 170 human consumers of contaminated shellfish (mainly *Perna perna* mussels (Proença et al., 2007a; Rosa and Philippi, 2009)) and led managers and regulators to issue a first-time ban for bivalve mollusk harvesting and commercialization. Recurrent small to medium-scale *Dinophysis* blooms in Brazil have been reported along the coasts of Paraná and Santa Catarina states since then (Mafra et al., 2015a, 2015b). In many cases, episodes of bivalve contamination have been reported based on Diarrheic Shellfish Poisoning (DSP) mouse bioassays (Proença, 1998; Proença et al., 2007a). Additionally, diarrheic toxins such as okadaic acid (OA) and their congeners *Dinophysis*toxins (DTXs) have been detected by chemical analytical methods in plankton and marine fauna (Mafra et al., 2015a; Proença, 1998). The current Brazilian national monitoring program for harmful algae and phycotoxins uses bivalves (especially brown mussels, *Perna perna*) as sentinel organisms for the presence of toxins in shellfish farming areas, and harvesting bans are issue anytime the regulatory toxin levels are surpassed, i.e. 160 $\mu g.Kg^{-1}$ in the case of diarrheic toxins (BRASIL, 2012a).

Dinophysis spp. are a recurrent threat to shellfish aquaculture areas worldwide (reviewed by Reguera et al., (Reguera et al., 2014)), where bloom initiation depends not only on favorable abiotic conditions, but also on the availability of ciliate prey (Moita et al., 2016). Mixotrophy via the sequestration and retention of plastids from the ciliate *Mesodinium* cf. *rubrum* is now considered a key process enabling the development of *Dinophysis* populations both in the laboratory and in the field (e.g. (Giménez Papiol et al., 2016a; Kim et al., 2012b)). Recent studies have focused on describing the feeding mechanism of *Dinophysis* spp., and elucidating the possible ecological roles of diarrheic toxins and other bioactives produced by the dinoflagellate (Giménez Papiol et al., 2016a; Luiz L. Mafra et al., 2016; Ojamäe et al., 2016). A number of nutritional and trophic aspects related to toxic *Dinophysis* spp. blooms, such as small-scale interactions with *M.* cf. *rubrum* and possibly with other prey items in the field, however, remain unclear.

During *Dinophysis* blooms, lipophilic toxins can be transferred via several trophic pathways (Jiang et al., 2007). Toxins can be accumulated not only by bivalves, but also by polychaetes and ascidians (Reizopoulou et al., 2008), fish (Mafra et al., 2014; Sipiä et al., 2000), octopuses (Mafra et al., 2015a) and crabs (Jiang et al., 2006; Vale and Sampayo, 2002). *Dinophysis* toxins may also be related to the death of monk seals off the coast of the western Sahara (Hernández et al., 1998), although the implications of toxin incorporation for marine organisms remain poorly known. Understanding of small-scale trophic relationships underlying the initiation and development of *Dinophysis* blooms, as well as the fate of diarrheic toxins in marine food webs, are essential for the evaluation of their associated risks. The main objectives of this study are to a) determine the diel vertical distribution of *Dinophysis* spp. and their prey in a shallow inlet, and b) quantify the levels of lipophilic toxins present in seston and in marine organisms representative of different trophic levels, during a massive bloom of the *Dinophysis acuminata* complex in southern Brazil.

Materials and methods

Study area

Armação do Itapocoroy is a shallow [mean depth = 8 m; maximum (max.) = 15 m] inlet in Santa Catarina State, southern Brazil (S 26° 47', W 48° 37'). Surrounded by hills (up to 250-m high), its geographic SE-NE orientation provides natural shelter from prevailing waves and winds, especially the stronger ones coming from the south. Due to these favorable attributes, Armação do Itapocoroy (Fig.1) harbors the major marine aquaculture (~360 hectares) operations in the country, mainly used for the cultivation of mussels, but also of oysters and scallops. The location experiences semi-diurnal, micro-tidal cycles and is affected by the Itajaí-Açu River plume, that maintains high levels of local primary production and brings high loads of suspended particulate matter (SPM) mainly during rainy periods such as the austral summer (December-March) (Schettini et al., 2005, 1999; Trochimczuk-Fo and Schettini, 2003).

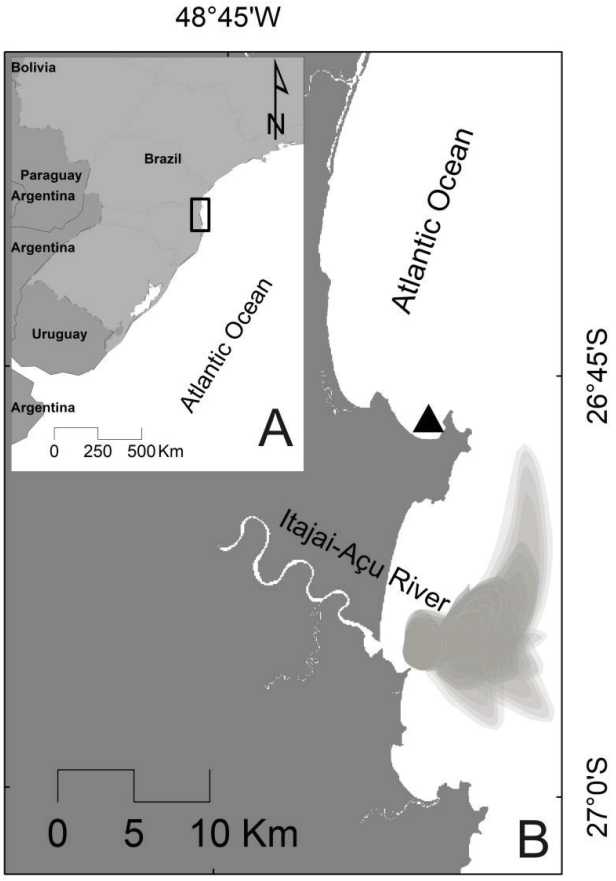

Figura 11 - Cap 3: Fig. 1 – Map showing the location of the study area, Armação do Itapocoroy inlet (triangle in (B)), on the southern coast of Brazil (rectangle in (A)). A schematic representation of the prevailing direction and influence area of the Itajaí-Açu River plume is also presented, based on Trochimczuk-Fo and Schettini (2003).

Sampling design

An intensive sampling effort was conducted between late May and mid-June 2016, when the Santa Catarina coastal zone was affected by a dense *Dinophysis* bloo*M.* Seawater sampling was carried out from a floating platform, anchored at a depth of 4.5 ± 0.5 m and deployed 200-m from the low tide level. Samples (~1.5 L) were taken every meter along a vertical profile, from the surface to the bottom, using a manual diaphragm pump (Emifran®, EN-470) equipped with anti-reflux valves and coupled to a 20 mm-diameter hose. Daily samples were taken over twelve days, followed by two sampling operations after 3- and 10-day intervals of the 12th sampling day. Additionally, single depth-integrated water samples (2.0 L) were taken with a 5-m long hose, to compare the efficiency of both sampling strategies for monitoring of extreme bloom events. Plankton net (20-µm mesh size) samples were also collected, and water temperature and salinity were measured along a vertical profile, at 0.5 m intervals from surface to bottom, using a multiparameter YSI Professional Plus probe. Secchi depth was used to estimate water column light penetration or transparency.

In parallel, bivalve mollusks (*Perna perna; n >5*), gastropods (*Stramonita haemastoma; n >2*), barnacles (*Megabalanus tintinnabulum; n >10*), amphipods (Caprellidae; ~10 g of wet weight), crabs (*Pilumnus spinosissimus; n >3*), shrimp (*Xiphopenaeus kroyeri; n >5*), polychaetes (*Pseudonereis palpate; n >3*) and fish (*Hypleurochilus fissicornis* – Blenniidae; n >2) were manually collected from raft mooring cables at 0-1 m depth, for quantification of the toxin levels incorporated in their tissues. The species collected and sampling frequency depended on their availability in the environment during the study period. Whenever possible, at least two individuals of each species were collected, packed in 50 mL plastic tubes and immediately immersed in an ice bath until return to the laboratory. Before freezing, soft tissues of mussels, barnacles and gastropods were removed from their shells. Fish muscles (flesh) and viscera were dissected and individually stored at -18ºC. The remaining organisms were frozen and analyzed whole.

Sample processing

Water samples: Aliquots (300 mL) of both depth-discrete and integrated samples were fixed with 1% Lugol's iodine solution, and used for quantitative phytoplankton analysis. Plankton net samples, fixed with a 4% formalin solution (final concentration), were used for analysis of cell morphology and

phytoplankton identification. In the laboratory, additional 300-mL aliquots of each sample were gently vacuum-filtered in duplicate and immediately frozen. One fiberglass filter (Marcherey-Nagel® 85/70BF; 47 mm diameter and 0.45 µm nominal retention capacity) was allocated for the analysis of photosynthetic pigments, and the second one to determine the amount of lipophilic toxins contained in SP*M.* In an ice bath, each filter sample was soaked in HPLC grade methanol (99.5%) and exposed to an ultra-sonic probe (Cole Parmer, CPX130) for 30 s. The extract was then passed through a 13 mm x 0.22 µm PVDF syringe filter (Analitica®) to remove any cell debris, and the filtrate collected into plastic microtubes (1.5 mL), which were maintained frozen. Aliquots (400 mL) of the filtrate from depth-integrated samples collected from May 1 on were stored frozen in plastic bottles for future spectrophotometric determination of dissolved inorganic nutrient (DIN) concentrations.

Samples of marine fauna: The protocol for toxin extraction was adapted from the official analytical method harmonized by the European Union (EURLMB, 2015). Methanol (99.5%; HPLC grade) was added in the ratio of 1:9 (v:v) to 1.0 ± 0.5 g of homogenate from selected tissues or whole body, exposed to an ultra-sonic probe (Cole Parmer, CPX130) until complete tissue disruption, and centrifuged at 2000 × g for 10 min. The supernatant was filtered through a 0.22-µm syringe filter directly into a 2.0-mL glass vial, and kept frozen for the analysis of diarrheic toxins in their free for*M.* Subsequently, 1-mL aliquots of the extract were subjected to alkaline hydrolysis by the addition of 2.5 M sodium hydroxide in a 76 °C thermal bath for 40 min, followed by the addition of 2.5 M hydrochloric acid to neutralize the solution and convert the conjugated (metabolized) toxins into their free toxin forms. The amount of conjugated toxins was obtained by subtracting the concentration of free toxins initially measured in the non-hydrolyzed extract from the total concentration of toxins obtained in the hydrolyzed extract.

Phytoplankton enumeration

Counting of *Dinophysis* spp. and *M.* cf. *rubrum* cells was performed using a 20-mL aliquot of the Lugol-fixed sample, after settling the particles for 24 h in a Utermöhl chamber (Edler and Elbrächter, 2010). Cell counting was then performed by scanning the whole chamber under an inverted optical microscope

at 200x magnification (limit of detection, LOD: 50 cell.L^{-1}). Other phytoplankton groups (total cryptophytes, diatoms, and dinoflagellates) were quantified in volumes ranging from 10 to 20 mL (depending on sample turbidity) by counting all cells contained in 5-10 random microscope fields of view (LOD: 2100–8400 cell.L^{-1}) after 24-h settlement. Additionally, a minimum of 100 cells of *Dinophysis* spp. were picked with a micropipette from *in natura* plankton net samples at the bloom apex (June 2 and 3; n = 5 samples each day), and placed into 1.5-mL plastic microtubes containing methanol 99.5% (HPLC grade) for determination of the toxin cellular quota.

Analysis of photosynthetic pigments by liquid chromatography (HPLC-DAD)

The methanolic extracts were injected (100 µL) into a liquid chromatography system (Hitachi® Chromaster), composed of a quaternary gradient pump, an automatic thermostat injector, a column oven (set at 40 °C) and a photodiode detector (DAD). Samples were eluted in a mixture of (A) methanol:acetone:pyridine (50:25:25) and (B) acetonitrile:acetone (80:20), at 1.0 mL min^{-1}. The proportion of B increased from 0 to 40% in 18 min, and then to 100% within the following 4 min of the analysis, remaining at 100% for an extra 16-min period before returning to the initial conditions (0% B) in the last 2 min (40 min in total). Pigment identification was performed by evaluating their retention times after the chromatographic separation in a Waters Symmetry® C8 column (150×4.6 mm, 3.5 µm particles), as well as their absorbance spectrum (350-750 nm scan), according to the methodology described by Zapata et al. (2000). The chlorophyll-*a* concentration was calculated using a linear regression obtained from successive dilutions of the analytical standard (Sigma-Aldrich) (0.8, 1.6, 3.1, 6.2, 12.5, and 25 ng mL^{-1}) with coefficient of determination higher than 98% (r^2> 0.98).

Spectrophotometric analysis of dissolved inorganic nutrients

Aliquots (25 mL) of the filtrate samples were used to determine the concentrations of phosphate ($P\text{-}PO_4^{3-}$), nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$), ammonium ($N\text{-}NH_4^+$), and silicate (SiO_2^-) using colorimetric methods (Grasshoff et al., 1999). The concentrations were determined from a linear regression

obtained from successive dilutions of the respective analytical standards (coefficient of determination, $r^2 > 0.90$).

Analysis of photosynthetic pigments by LC-DAD

The methanolic extracts were injected (100 µL) into a liquid chromatography (LC) system (Hitachi® Chromaster), composed of a quaternary gradient pump, an automatic thermostat injector, a column oven (set at 40 °C) and a photodiode detector (DAD). Samples were eluted in a mixture of (A) methanol:acetone:pyridine (50:25:25) and (B) acetonitrile:acetone (80:20), at 1.0 $mL.min^{-1}$. The proportion of B increased from 0 to 40% in 18 min, and then to 100% within the following 4 min of analysis, remaining at 100% for an extra 16-min before returning to the initial conditions (0% B) for an additional 2-min period (40 min in total). Pigment identification was performed by evaluating retention times after the chromatographic separation in a Waters Symmetry® C8 column (150×4.6 mm, 3.5 µm particles), as well as the absorbance spectrum (350-750 nm scan), following methods of Zapata et al. (Zapata et al., 2000). The chlorophyll-*a* (chl-*a*) concentration was calculated using a linear regression obtained from successive dilutions of the analytical standard (Sigma-Aldrich) (0.78, 1.56, 3.12, 6.25, 12.50, and 25.00 $ng.mL^{-1}$) with $r^2 > 0.98$.

Analysis of diarrheic toxins by LC-MS/MS

Toxins were measured using liquid chromatography (LC) system (Agilent Technologies® 1260) coupled to a triple quadrupole mass spectrometer, MS (AB Sciex® qTRAP 3200) equipped with a turbo ion spray ionization source, following the EURLMB protocol (EURLMB, 2015). Briefly, 5 to 15 µL of each sample were eluted by the mobile phase, consisting of a mixture of (A) 100% ultra-pure water and (B) 95% acetonitrile, both with the addition of ammonium formate (2 mM) and formic acid (50 mM). At a 0.3 $mL.min^{-1}$ flow rate, the initial proportion of 80:20% (A:B) increased to 100% B during the first 8 min of analysis, thus remaining for 3.5 min, and returning to the initial condition by the end of the analysis (13 min). Compounds were separated on a C18 column (Agilent Poroshell®, 50×2.1 mm, 2.7 µm particles), maintained at 20 °C. Identification of individual toxins was achieved from their retention time and the mass spectra of the transition ions present in the samples in relation to the same parameters obtained for the

analytical standards. High-purity nitrogen, heated up to 500°C, was used as the nebulizing gas. The electron spray (ESI) ion source operated in negative mode, and toxins were scanned for transition ions (Q1→Q3) of characteristic mass/charge (m/z) ratios. Optimized MS parameters were selected for each toxin of interest (Table 1).

Table 4 - Table 1. Conditions of the tandem mass spectrometry system (MS/MS). Q1: quadrupole 1, Q3: quadrupole 3, DP: declustering potential, EP: entrance potential, CEP: collision cell entrance potential, CE: collision energy and CXP: collision cell exit potential

Toxins	Q1 (m/z)	Q3 (m/z)	DP (v)	EP (v)	CEP (v)	CE (v)	CXP (v)
OA	803.5	255.0	-129	-10	-40.1	-82	-2
OA	803.5	113.0	-129	-10	-41.5	-64	-2
DTX-2	803.5	255.0	-129	-10	-40.6	-64	-2
DTX-2	803.5	113.0	-129	-10	-41.5	-84	-2
DTX-1	817.5	255.0	-129	-10	-41.5	-62	-2
DTX-1	817.5	113.0	-120	-10	-51.7	-82	-2
DTX-3	1041.6	255.0	-129	-10	-47.9	-76	-2

Toxin quantification was carried out using an external standard from a calibration curve generated with certified reference material (IMB-NRC, Canada) dissolved in methanol for OA, and in mussel tissue matrix (CRM-DSP-Mus-b) for DTX-1. Quantification of OA was based on the equation obtained by fitting a linear regression ($r^2 > 0.95$) to the following concentrations: 3.49, 13.96, 55.86, and 223.44 $ng.mL^{-1}$.

Data analysis:

Graphs were constructed in SigmaPlot® v11.0, using the statistical package for preliminary analysis. Data were statistically analyzed with R studio software (R Core Team, 2017), using the Kruskal-Wallis non-parametric test (H) followed by the Dunn test (with the software package dunn.test; (Dinno, 2017)) for comparative analyses within and among water column depths, between discrete and integrated samples, and for analysis of temporal variation. Principal components analysis (PCA) (with the FactoMiner package; (Lê et al., 2008)) allowed quantification of the degree of association among water temperature,

Secchi depth, salinity, numerical abundance of main taxonomic groups, concentration of dissolved inorganic nutrients and seston toxin content.

Results

Plankton and toxins in the water column

Depth-averaged water temperature and salinity decreased gradually during the first half of the bloom period, from May 26 to June 3, when the minimum salinities were recorded (mean ± standard deviation (SD) = 24.2 ± 0.55 ; n = 6). The water temperature continued to decrease thereafter, attaining a minimum of 16.8 ± 0.1 °C on the last sampling day, June 16 (Fig. 2A, B). Secchi depth (Fig. 2C, D) ranged from 1.8 to 2.8 m during the first half of the bloom and then gradually increased, attaining up to 3.6 m by the end of bloo*M.* Chlorophyll-*a* concentrations were relatively low (0.47 ± 0.17 SD $mg.m^{-3}$) throughout the study, and reached a maximum of 1.1 $mg.m^{-3}$ on June 3rd, coinciding with the maximum peak of *Dinophysis* abundance. Decreasing concentrations of mean DIN (± SD), especially those of nitrate (2.3 ± 0.9 µM) and ammonium (2.5 ± 0.7 µM) (Fig. 2 E), were associated with a concurrent decrease in salinity and temperature, and an increase in the abundance of *M.* cf. *rubruM.* Silicate and phosphate exhibited a marked increase during later stages of the bloom and attained the highest concentration range (39.6–88.7 µM and 1.3–6.3 µM, respectively) by the end of the study period (Fig. 2F).

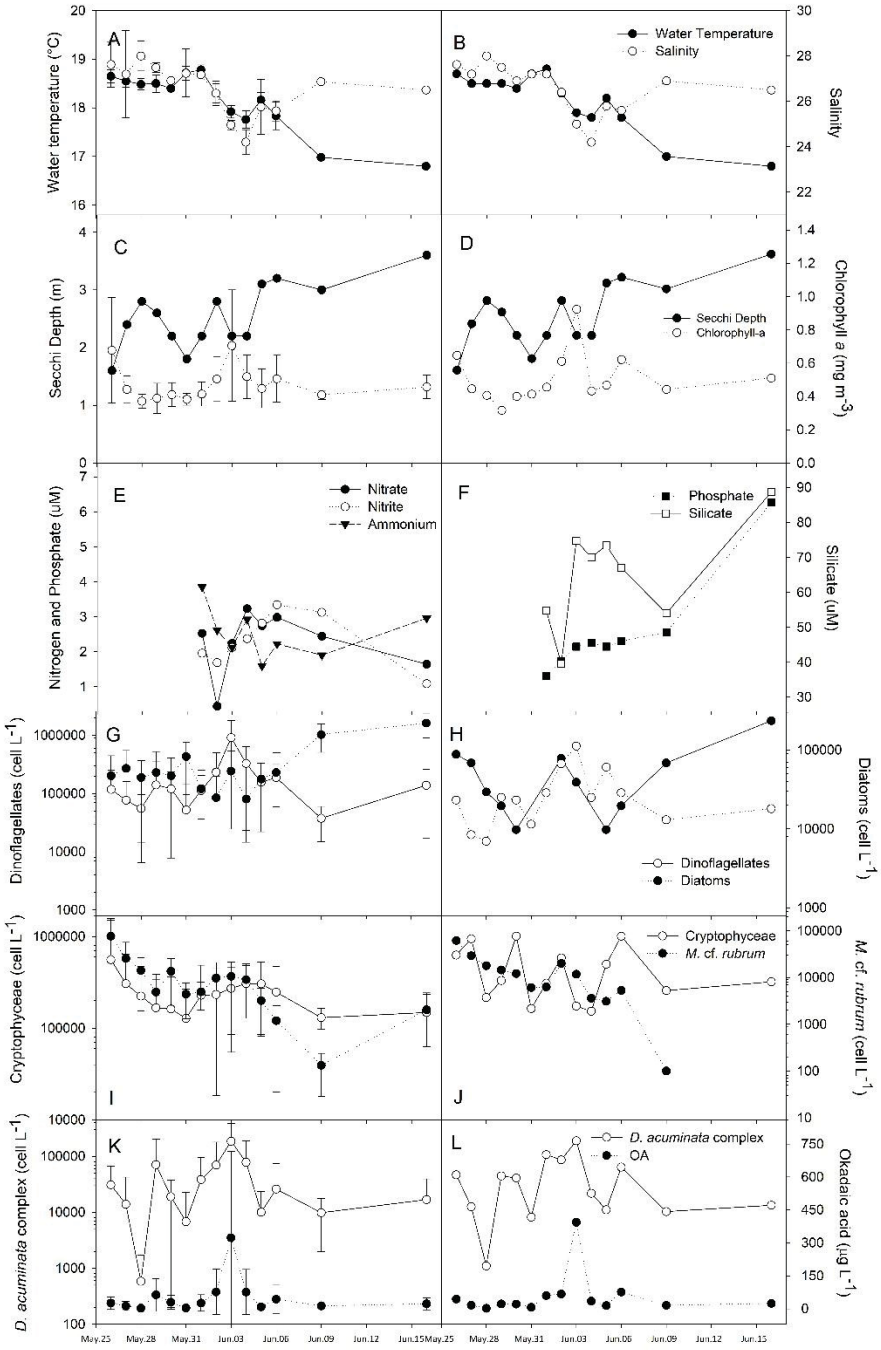

Figura 12 - Cap 3: Fig. 2 – (A, C, G, I, K) Average values (± standard deviation; n = 6) of depth-discrete measurements, and (B, D, E, F, H, J, L) single depth-integrated measurements (taken with a hose extending from the surface to the bottom) for: (A, B) water temperature (ºC) and salinity ; (C, D) Secchi depth (m) and chlorophyll-a concentration (mg.m-3); (E, F) concentration of dissolved inorganic nutrients (µM); (G, H) numerical abundance (on log-scale) of dinoflagellates and diatoms (cells.L^{-1}); (I, J) abundance of cryptophyceans and Mesodinium cf. rubrum (cells.L^{-1}); (K, L) abundance of the Dinophysis acuminata spp. complex (cells.L^{-1}) and concentration of free okadaic acid (OA) in suspended particulate matter (µg.L^{-1})..

The beginning of the bloom (May 26th) was marked by an intense superficial saline stratification and water temperatures around 18 ºC (Fig. 2). After 2-3 days, the saline stratification was disrupted and the temperature increased 1.0 ºC, decreasing thereafter to a minimum of 17 ºC by the 10th day (June 4th), when

the water column became saline-stratified again (Fig. 2). Water temperature ranged from 17 to 19.5 ºC and salinity from 23 to 29 PSU over the course of time. The relatively high transparency values (>2.0 m) indicate that the euphotic zone always reached the bottom in the sampling area.

Diatom abundance remained at low to moderate levels (<9×10^4 cells.L^{-1}) during the first half of the bloom, rising up to 19 ± 6.2×10^4 cells.L^{-1} by the end of the sampling period, when they finally dominated the micro-phytoplankton assemblage (Fig. 2H). Dinoflagellates were detected at cell densities comparable to those of diatoms during the first half of the study, attaining a maximum of 90 ± 31.9×10^4 cells.L^{-1} and becoming dominant over diatoms on June 3 (Fig. 2H). *Dinophysis* species found during this bloom included the taxonomic complex composed by *D. acuminata* and *D. ovum* (referred to as *D. acuminata* complex hereafter), and *D. caudata*. This last species was frequently observed in plankton net samples during the first half of the bloom, although no cells were detected in most cell counts (LOD: 50 cells.L^{-1}), except on May 28 and 30 (100 cells.L^{-1}; not shown). The *Dinophysis acuminata* complex comprised the most abundant dinoflagellate cells and the main component of the total micro-phytoplankton assemblage throughout the study. Their depth-averaged cell density increased from 7.1 ± 13.4×10^4 cells.L^{-1} on the first sampling day to 18.7 ± 20.1×10^4 cells.L^{-1} (max. 43×10^4 cells.L^{-1} at 1.0 m depth) on June 3rd (Fig. 2K), coinciding with a gradual decrease in the abundance of the ciliate *M.* cf. *rubrum* (Fig. 2I). At the beginning of the bloom, average cell densities of cryptophyceans decreased at a rate comparable to that of *M.* cf. *rubrum*, and then increased slightly by the mid-bloom period, when *M.* cf. *rubrum* abundance reached minimum values (Fig. 2I).

Water temperature (Kruskal-Wallis test statistic H = 69.4; p = 0.01), Secchi depth (H = 79.0; p = 0.01), salinity (H = 55.5; p = 0.01) and abundance of diatoms (H = 34.3; *p* = 0.01) all varied significantly during the study period. When depth layers were compared over time, however, there was no detectable difference in water temperature (H = 0.24; p = 0.99) or diatom abundance values (H = 6.6; p = 0.25) over time at any specific depth. The depth layer marking salinity stratification (H = 14.8; p = 0.01) ranged from 2 to 3 m over the course of the bloo*M.* Although peaks in the abundance of targeted taxa were clearly identified in time and space/depth, there were no statistically significant differences in the cell density of dinoflagellates, cryptophyceans, *M.* cf. *rubrum* and the *D.*

acuminata complex, or in the concentrations of chl-*a* and OA in SPM over time. Chlorophyll-*a* concentrations ($H = 45.0$; $p < 0.01$), and the abundance of dinoflagellates ($H = 51.7$; $p < 0.01$), cryptophyceans ($H = 56.4$; $p < 0.01$), *M.* cf. *rubrum* ($H = 31.6$; $p < 0.01$), and the *D. acuminata* complex ($H = 53.4$; $p < 0.01$) all differed significantly between 0-2 m and 3-5 m depth, with higher values found in the upper water layer. In general, depth-integrated samples (those taken with a hose extending from surface to bottom), yielded very similar values to those calculated as the average of depth-discrete measurements, except for the abundance of cryptophyceans ($H = 20.3$; $p < 0.01$), which was typically greater when estimated from integrated samples (Fig. 2I, J).

The onset of the bloom (on May 26) was marked by pronounced surface stratification in salinity and water temperatures around 18 ºC (Fig. 3). After 2-3 days, salinity stratification was disrupted and the temperature increased by 1.0 ºC, decreasing thereafter to a minimum of 17 ºC by the 10th day (June 4), when the water column again became salinity-stratified (Fig. 3). Water temperature ranged from 17 to 19.5 ºC and salinity from 23 to 29 over time. The relatively high Secchi-depth values (>2.0 m) obtained during the study indicate that the euphotic zone always reached the bottom in the sampling area.

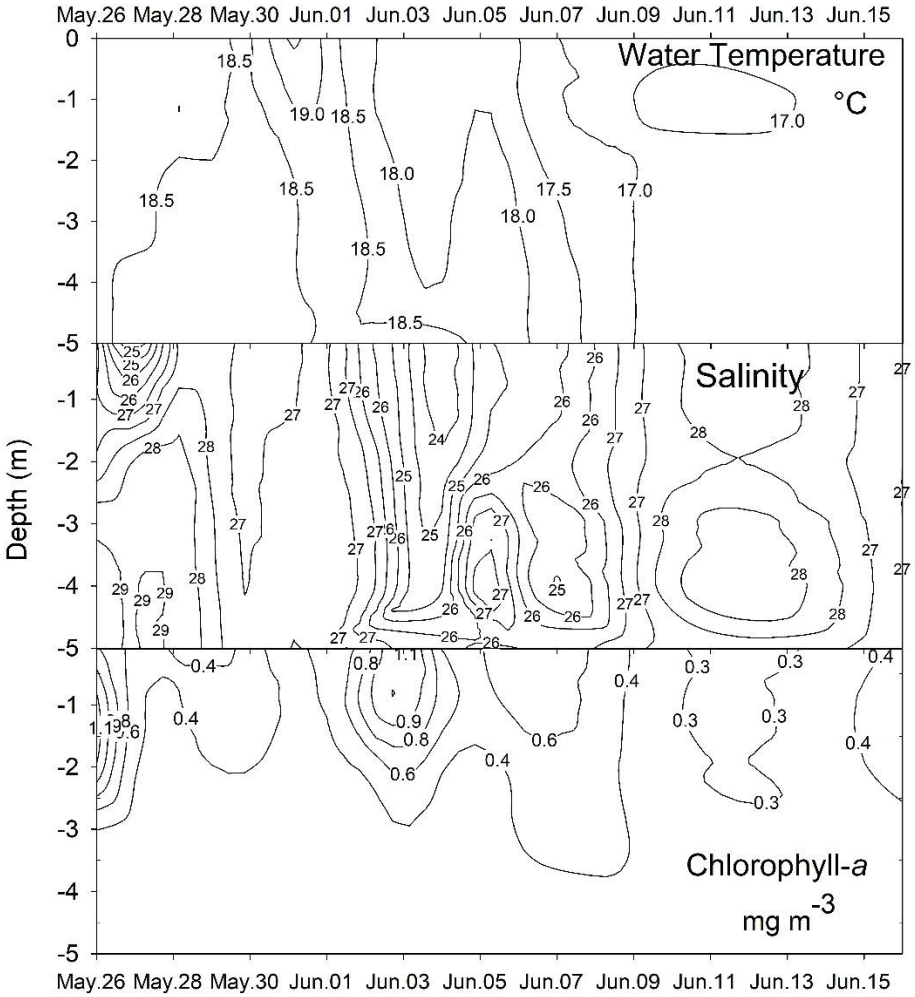

Figura 13 - Cap 3: Fig. 3 Interpolated depth-discrete measurements of water temperature (ºC), salinity, and chlorophyll a concentration (mg.m^{-3}), over the course of the study..

Chlorophyll-*a* concentrations were higher during periods of salinity stratification. Values ≥1.0 mg.m^{-3} were attained on two occasions, on the 1st and the 9th day of sampling (May 26 and June 3), with a third peak (>0.6 mg.m^{-3}) a few days later (Fig. 2D, 3). In all cases, higher concentrations were restricted ($H = 0.45$; $p < 0.01$) to the surface layer (0–2 m). Likewise, the abundance of the main taxa investigated – cryptophyceans, *M.* cf. *rubrum* and *Dinophysis* – also varied vertically, exhibiting higher values in the upper water layer. Maximum values for cryptophyceans and *M.* cf. *rubrum* (>500×10^4 and 18×10^4 cell.L^{-1}, respectively) were measured on the first day of sampling. During subsequent days, their abundance decreased concomitantly with a rapid increase of the *D. acuminata* complex cell density, reaching >20×10^4 cells.L^{-1} on May 29 and >40×10^4 cells.L^{-1} on June 3, one week following the initial peak in *M.* cf. *rubrum*

abundance (Fig. 4), and coincident with the second episode of salinity stratification. Higher cell abundances of the *D. acuminata* complex were restricted to the surface layer (>2 m), where concentrations of free-OA >600 µg.L^{-1} were simultaneously detected in the SPM (>0.45-µm particles). During the period of maximum dinoflagellate cell density, the OA cellular quota, as measured from individually-picked cells of the *D. acuminata* complex ranged from 48 ± 31 SD pg.cells^{-1} (n = 137-208 cells per sample) on June 2 to 143 ± 93 pg.cell^{-1} (n = 141-220) on June 3.

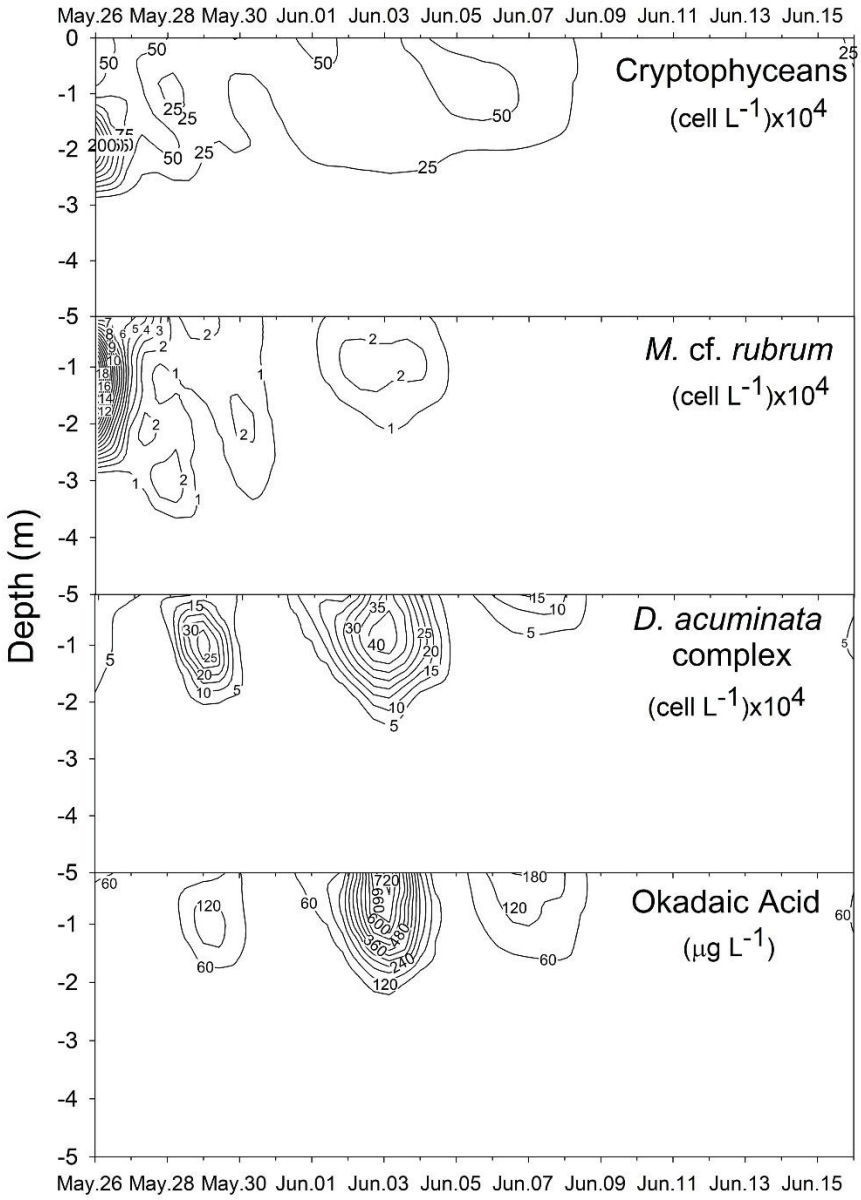

Figura 14 - Cap 3: Fig. 4 – Depth profile of the cell abundance of the main plankton taxonomic groups (cell.L^{-1}x10^{4}) (top three panels) and the concentration of okadaic acid (µg.L^{-1}) in suspended particulate matter (bottom panel) during the study period.

Diarrheic toxins in marine fauna

All selected marine faunal components accumulated detectable OA levels during the study. Toxin levels were directly associated with the presence of *D. acuminata* complex cells in the water column; maximum values depended on the species and trophic position of the organisms. Suspension-feeding mussels and barnacles were the first to accumulate detectable levels of OA in their tissues and the only ones to contain detectable toxin levels during the entire sampling perio*D.* Mussels accumulated the highest OA concentrations among all organisms analyzed, with toxin levels gradually increasing following an increase in cell density of the *D. acuminata* complex. They attained a maximum of 549.6 µg total OA.Kg^{-1} (wet tissue weight) on June 4 (Fig. 5), only one day after the peak abundance of the dinoflagellate. Polychaete worms (max. = 164.5 µg total OA.Kg^{-1}), amphipods (max. = 153.7 µg total OA.Kg^{-1}) and gastropods (max. = 111.6 µg total OA.Kg^{-1}) also retained relatively high toxin amounts, but not before the mid-bloom stage. Amphipods and to some extent fish (max. = 56.2 µg total OA.Kg^{-1}), remained contaminated for shorter periods, i.e., only when OA concentrations in the SPM were maximal (early to mid-bloom period). In contrast, gastropods, polychaetes, crabs (max. = 33.3 µg total OA.Kg^{-1}) and shrimp (max. = 7.2 µg total OA.Kg^{-1}) accumulated detectable toxin levels from the mid- to late bloom stage, after OA peaked in suspension (Fig. 5).

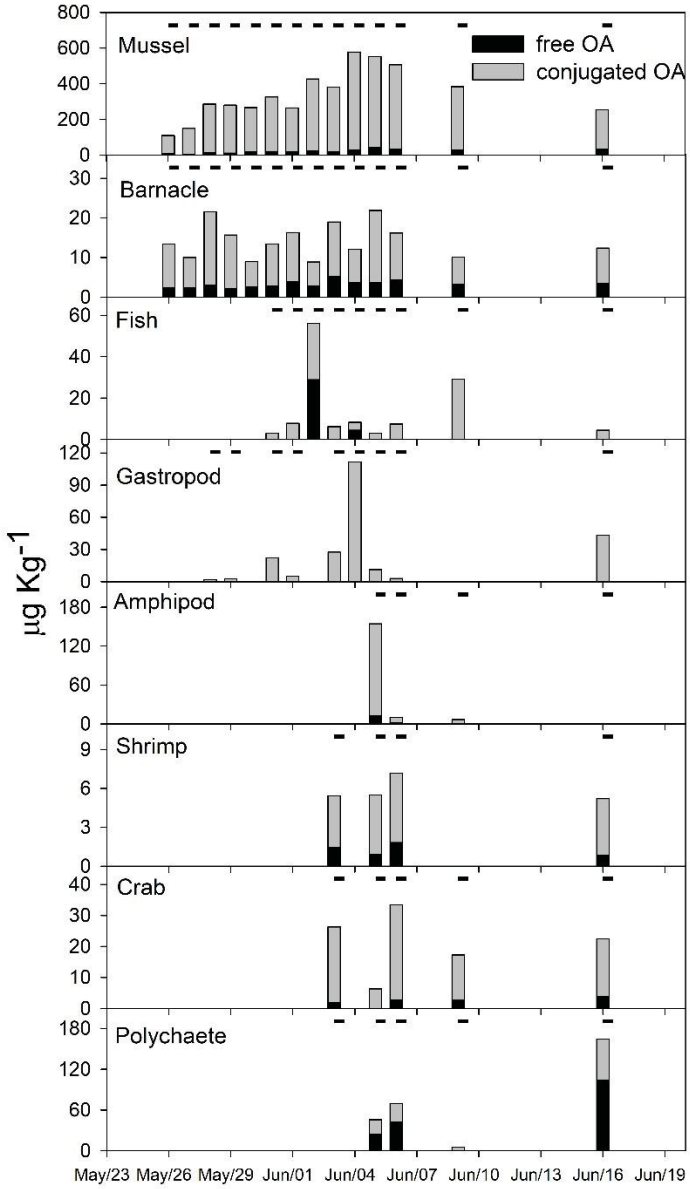

Figura 15 - Cap 3: Fig. 5 – Concentration of okadaic acid (OA, µg.kg^{-1}), in its free (black bars) and conjugated (gray bars) forms, accumulated in different marine organisms during the bloom of the Dinophysis acuminata spp. complex. Dashes above the composite bars denote the sampling dates when each marine organism was available..

Fish and polychaetes accumulated the greatest proportions of OA in its free for*M.* The proportion of free-OA was slightly higher in fish (54 ± 7%) at the peak of the bloom, but still did not match that of polychaetes (64 ± 5%). The latter seemed to possess the least efficient detoxification mechanism, given the high proportions of free-OA and the constantly increasing total OA levels measured (Fig. 5). Conversely, the conjugated forms of OA were dominant in all other organisms, including barnacles (76 ± 7% SD), shrimp (79 ± 5%), amphipods (87

± 5%), crabs (90 ± 7%), mussels (94 ± 2%) and gastropods, which never accumulated detectable levels of free-OA (i.e., exhibited 100% of OA as conjugated forms).

Correlations

As assessed by principal component analysis, salinity was strongly and inversely correlated with both the abundance of the *D. acuminata* complex and the concentration of OA in suspension (Fig. 6). This grouping was also inversely, but less obviously associated with ammonium concentration and the abundance of cryptophyceans, and even less obviously with Secchi depth, phosphate concentration and the abundance of diatoms (Fig 6). These last three variables, as well as the concentration of silicate, were inversely correlated to *M.* cf. *rubrum* abundance, which, in turn, was strongly and directly associated with the abundance of cryptophyceans and the concentrations of nitrite and nitrate.

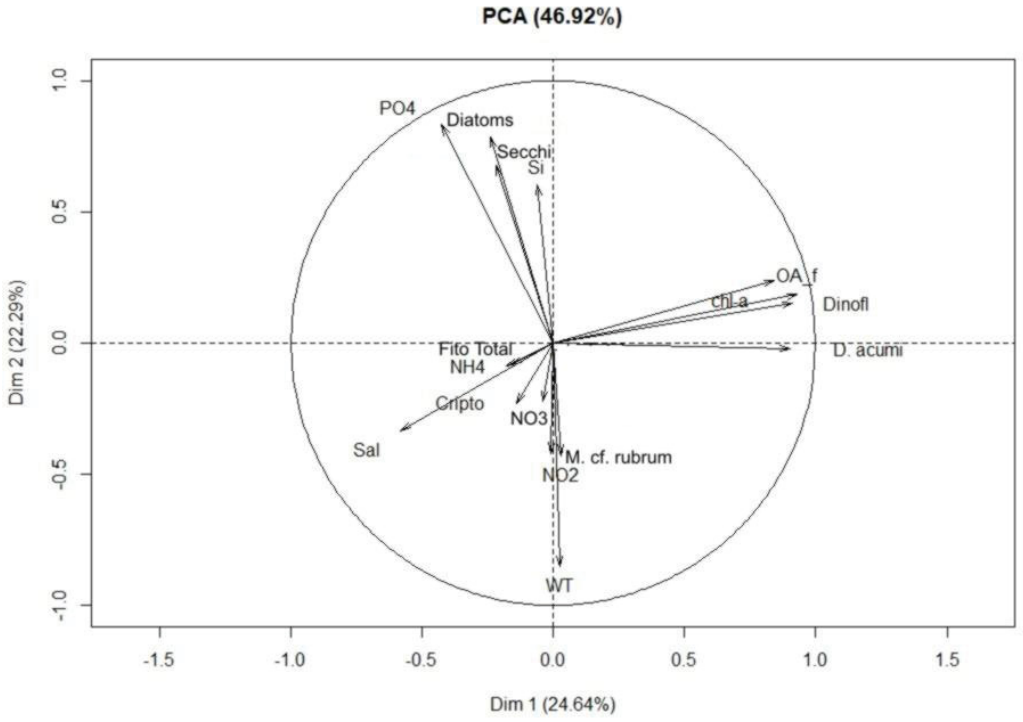

Figura 16 - Cap 3: Fig. 6 – Principal Component Analysis (PCA) of discrete-depth measurements of the following variables: water temperature (WT), Salinity (Sal), Transparency (Secchi depth), Chlorophyll-a (chl-a), Diatoms (Diatoms), Dinoflagellates (Dinofl), Cryptophyceans (Cripto), total micro-phytoplankton (Fito Total), Mesodinium cf. rubrum (M. cf. rubrum), D. acuminata complex (D. acumi), free okadaic acid (AO_f), Phosphate (PO4), Nitrate (NO3), Nitrite (NO2), Ammonium (NH4), and Silicate (Si) concentrations..

Discussion

Bloom development and trophic relationships

In late May 2016, an episode of marked salinity stratification was associated with the onset of what can be considered the most intense bloom of the *Dinophysis acuminata* complex ever recorded along the Brazilian coast. The bloom only lasted for a few weeks along the coast of Santa Catarina State. It was then transported northward to Paraná State, where it reached even higher cell densities, causing massive contamination of marine fauna and intoxication of human seafood consumers (Luiz Laureno Mafra et al., 2016). Although results of the present study indicated that bivalves were also contaminated with unsafe OA levels in Santa Catarina, actions taken in the context of the local HAB monitoring and management program prevented cases of intoxication in this region. More importantly, however, the present study also documents the accumulation of diarheic toxins in several other marine organisms associated with farmed mussels, some of them for the first time, indicating that multiple toxin vectors and transfer routes should be considered during massive *Dinophysis* blooms.

Blooms of *Dinophysis* spp. are usually associated with marked thermohaline stratification of the water column (Aissaoui et al., 2013; Alves-de-Souza et al., 2014; Díaz et al., 2011; Villalobos et al., 2015). The ciliate prey of *Dinophysis* spp., frequently reported as *Mesodinium rubrum*, usually benefits from vertical water stratification as well (Crawford and Lindholm, 1997; van den Hoff and Bell, 2015), although blooms of the ciliate may also occur along horizontal thermohaline gradients in shallow estuaries (Johnson et al., 2013).

About one week preceding the maximum *Dinophysis* cell density recorded in Armação do Itapocoroy inlet during this study, high abundances of *M.* cf. *rubrum* and their cryptophycean prey (10^5 to 10^6 cells.L^{-1}, respectively) were observed in the upper water layer associated with lower salinities and strong stratification at 2 m depth. On the following 4-5 days, the abundance of cryptophyceans decreased rapidly, followed by a more gradual decrease in *M.* cf. *rubrum* cell density, as the abundance of cells belonging to the *D. acuminata* complex began to increase in the same surface layer. One week later, a second cryptophycean-ciliate-*Dinophysis* succession cycle occurred once water became stratified again. Although daily variations in the abundance of these three taxa may be partially

linked to local advection, what was not the subject of this study, the succession pattern reported herein confirms that the trophic relationships documented in prior laboratory observations (Giménez Papiol et al., 2016a; Kim et al., 2012a; Luiz L. Mafra et al., 2016; Stern et al., 2014a; Wisecaver and Hackett, 2010)may also occur on a similar temporal scale under natural field conditions and sustain massive blooms of the toxic dinoflagellate. In other occasions (i.e. under lower availability of *M.* cf. *rubrum* cells), alternative prey items may provide *Dinophysis* spp. with an additional source of nutrients, as suggested for *D. caudata* preying upon the benthic ciliate *Mesodinium coatsi* (Kim et al., 2015).

The development of *Dinophysis* blooms in other geographical areas may also be linked to the intrusion of less saline water masses and/or to disturbances in physico-chemical water column structure, although the underlying processes might be different and sometimes occur on a wider spatio-temporal scale. Blooms may thus be either associated to upwelling, as verified in Sweden (Lindahl et al., 2007), Galicia (Spain) and Portugal (Moita et al., 2016), or to river plumes, as found in Tunisia (Aissaoui et al., 2013) and Scotland (Whyte et al., 2014b). They may also be associated with seasonal changes in wind patterns and the precipitation regime such as those recorded in Ireland (Raine, 2014), Greece (Koukaras, 2004) and Argentina (Fabro et al., 2015; Sar et al., 2012). On the eastern coast of South America the water mass associated with the La Plata River plume promotes important large-scale changes in the physico-chemical characteristics of the water column along the coasts of NE Argentina, Uruguay and southern Brazil during fall and winter (Möller et al., 2008; Piola et al., 2008), when massive *Dinophysis* blooms are usually observed in this region (S. Méndez et al., 2016a, 2016b). This suggests that the La Plata water plume (PWP) may be one of the main factors controlling the development of large-scale *Dinophysis* blooms in southwestern Atlantic coastal waters.

Chlorophyll-*a* concentrations did not vary substantially over time in the present study, and did not attain values exceeding the historical average for the region (Ferreira et al., 2006; Proença, 2002). This could be explained by the uncommon prevailing phytoplankton succession, whereby one dominant taxon preys upon and acquires the plastids (and the pigments) from its precursor, rather than synthesizing its own pigment quota during a gradual competitive exclusion process. Whereas phosphate concentrations remained relatively high and even

increased during the final bloom stage, those of dissolved nitrogen compounds, notably ammonium and nitrate, decreased over the course of the bloom, especially during the period of maximum cell abundance of the *D. acuminata* complex.

Both water sampling strategies used in this study (single integrated samples and multiple depth-discrete sampling) allowed adequate tracking of bloom development, yielding similar abundance values for both the toxic dinoflagellate and its prey, *M.* cf. *rubruM.* Therefore, as demonstrated in other areas such as Spain (Escalera et al., 2012), depth-integrated sampling, undertaken with a hose extending from the surface to the bottom, provided a rapid and reliable early-warning tool in HAB monitoring and risk assessment in shallow waters affected by *Dinophysis* blooms along the southern coast of Brazil. However, special attention is required when applying the technique to ecological studies, as the abundance of cryptophyceans and perhaps other small-celled algal groups can be underestimated. Likewise, vertical migration and cell aggregation processes can be missed as a result of the "diluting" effect introduced by this sampling strategy. More importantly, adoption of *Dinophysis* cell abundance as early warning for DSP should be used conservatively, i.e., the threshold value should be kept cautiously low when integrated samples are used in HAB monitoring programs. One of the main ecological features of *Dinophysis* cells is their ability to aggregate in thin water layers, as reported in this and other studies (Farrell et al., 2012), such that toxin food web transfer may be heterogeneous throughout the water column.

Fate of diarrheic toxins during the bloom

Cells of the *D. acuminata* complex contained exclusively OA during the bloom described in this study, contrasting with a more complex toxin profile reported during previous blooms in Argentina (Aune et al., 2007; Villalobos et al., 2015) and Chile (Blanco et al., 2007b), where *D. acuminata* and *D. tripos*, the species involved in the blooms, produced pectenotoxin-2 (PTX-2) and DTX-1 in addition to OA. It is noteworthy that DTX-1 has been reported in different southern Brazilian estuaries when lower *Dinophysis* spp. cell abundances ($<2\times10^4$ cell.L^{-1}) occur, but rarely when only cells of the *D. acuminata* complex are detected, in which case OA usually becomes the single diarrheic toxin present (Mafra et al., 2014). Similarly, in late summer 2015, one year before the event reported in this

study, an extremely dense bloom of the *Dinophysis acuminata* complex affected the coast of Uruguay and only OA was detected by LC-MS/MS (S. *M.* Méndez et al., 2016). This further suggests that there may be interconnectivity between Uruguayan and southern Brazilian populations of the *D. acuminata* complex, perhaps driven by the northward transport of PWP from late summer to winter. This possibility remains to be addressed in future studies.

All marine organisms collected in the upper water layer (0-1 m) at the sampling site were consistently contaminated with varying amounts of OA. This is the first record of diarrheic toxin accumulation in amphipods (Caprellidae), shrimp (*X. kroyeri*), Nereidae polychaetes and blenny fish (Blenniedae). The only previous records of OA content in fish included carnivorous flounders, *Platichthys flesus* (Sipiä et al., 2000), and filter-feeding anchovies, *Cetengraulis edentulus* (Mafra et al., 2014). Results of the present study demonstrated that the combtooth blenny, *Hypleurochilus fissicornis,* can accumulate moderate OA levels in their viscera during *Dinophysis* blooms, but are able to rapidly eliminate the toxin. Therefore, this fish species may act as a temporary vector of diarrheic toxins for other species, including commercially important species of Serranidae and Lutjanidae, which prey upon small fishes like blennies (Froese and Pauly, 2017). *Hypleurochilus fissicornis* is widely distributed in the southwest Atlantic; adults feed primarily on isopods and amphipods (Menezes and Figueiredo, 1985), that were likely an important – although probably not the sole – toxin source for the fish during the bloom, as the peak in OA levels occurred later for amphipods than for *H. fissicornis* in the present study. Amphipods accumulated relatively high OA levels (up to ~150 µg OA.Kg^{-1}) at the peak of the bloo*M.* Although most amphipods are detritivores/scavengers, caprellids such as the ones sampled in our study are omnivorous and may feed not only on detritus, but also on microalgae, protozoans, smaller amphipods and crustacean larvae (Caine, 1991). Caprellids are frequent and abundant organisms associated with suspended mussel farms, living on substrates such as mussel sleeves and ropes, and may thus be important toxin vectors to several organisms that search for shelter and food within the mussel longlines in aquaculture areas.

Mussels accumulated the greatest OA levels during the bloom, exceeding by 4-fold the 160-µg.Kg^{-1} Brazilian regulatory seafood safety level (BRASIL, 2012b). *Perna perna* mussels are the sentinel species in HAB monitoring programs in

Brazil and, like other mussel species, are able to rapidly incorporate high levels of several marine biotoxins and contaminants (Brooks et al., 2012; Higman et al., 2007; Penna et al., 2006; Trainer and Hardy, 2015; Vale et al., 2008b). Indeed, along with barnacles, mussels were the only organisms exhibiting detectable OA levels during the entire sampling perio*D.* They consistently and promptly reflected the abundance of the *D. acuminata* complex (i.e., they attained maximum OA levels only one day after the peak in cell abundance). Considering the high *Dinophysis* cell abundance reported during this bloom, however, OA levels in *P. perna* were not as high as expected, what may be related to its fast toxin elimination rates as reported in previous laboratory experiments (Mafra et al., 2015b). Besides mussels, non-edible polychaetes (*P. palpata*) and amphipods were the only organisms to accumulate total OA levels approaching or surpassing this regulatory level in the present study. Toxin contents in barnacles were 8 to 48x lower – and less clearly related to *Dinophysis* cell density – than those of mussels. Barnacles colonize hard substrates, rocks, bivalve shells and mooring structures, living in clusters of around a dozen individuals that actively capture food particles from the surrounding water (Doyle et al., 1996). In the present study, barnacles were collected from the shells of the same mussels sampled for OA analysis, so that the differential toxin accumulation reported here for these two suspension-feeding taxa can only be attributed to distinct feeding mechanisms and toxin uptake/elimination capacity. The consistently greater proportions of conjugated OA in mussels (>90%) reflect their efficient mechanisms of toxin metabolism and elimination. Likewise, Caprellidae amphipods, small crabs (*P. spinosissimus*), shrimp (*X. kroyeri*) may have ingested toxins from the grazers or their organic matter produced (Maneiro et al., 2002b; Turner, 2014), and carnivorous gastropods (*S. haemastoma*) also accumulated very limited to undetectable free-OA levels. This finding at least partly suggests that these organisms ingested already metabolized (i.e., conjugated) toxin, either incorporated into mussel and barnacle tissues (gastropods, shrimp and crabs) or from detrital origin (in the case of amphipods and crabs). High toxin levels were found in seston during this study and in particles >60 µm (L. Mafra, unpublished data), suggesting that not only *Dinophysis* cells but also toxin-containing organic particles and zooplankton organisms may contribute to the transfer of diarrheic toxins along the foodweb.

Therefore, although zooplankton (e.g. copepods) may exert significant grazing impact and contribute considerably to control population growth of *Dinophysis* spp. (Kozlowsky-Suzuki et al., 2006), contaminated individuals will act as vectors of DSP-toxins to higher trophic levels.

Transfer of lipophilic toxins in the marine food web is still poorly understood. Although they represent only a small fraction of the sinking organic material, zooplankters such as the copepod *Temora longicornis*, might contribute in maintaining toxin availability for other organisms via production of toxic faecal pellets following ingestion of *Dinophysis* cells (Maneiro et al., 2002b). Inter- and intraspecific differences in the capacity of uptake and elimination of phycotoxins, as reported for suspension-feeding grazers such as oysters, clams and mussels (Armi et al., 2012; Dumbauld et al., 2009; García-Mendoza et al., 2014; Mafra et al., 2015b), may ultimately determine the bioavailability of these compounds for other organisms during and after a bloo*M.* In this study, polychaetes, which exhibited the highest proportions of free-OA and whose total OA levels continued to increase through the end of the sampling period, proved to be slow in eliminating OA. They may thus be an important toxin vector during late bloom stages, by prolonging toxin availability in the trophic web even after bloom termination.

To date, potential vectors of diarrheic toxins to human consumers have been restricted to several bivalve species (reviewed by FAO/WHO (FAO/WHO, 2016)), a couple of fish species (Mafra et al., 2014; Sipiä et al., 2000), octopuses (Mafra et al., 2015a) and crabs (Jiang et al., 2006; Vale and Sampayo, 2002). The present study indicates that seabob shrimp (*X. kroyeri*) can represent a novel vector for toxin transfer to humans during massive dinoflagellate blooms. Although these shrimp accumulated the lowest OA levels (≤7 $\mu g.Kg^{-1}$) of all investigated faunal species and thus cannot be classified as a risk for acute food intoxication among seafood consumers, frequent consumers of this valuable fishery resource may be chronically exposed to low toxin levels during prolonged blooms. Seabob shrimp catches may reach 170 tons per year only in the Armação do Itapocoroy area (Branco, 2005), our study site, and the seabob shrimp fishing season coincides with the usual period of *Dinophysis* blooms in southern Brazil (winter to spring; (Alves et al., 2018)). Additionally, shrimp and, to some extent crabs and blenny fish, are highly motile. Their frequent vertical

migration throughout the water column or on mussel ropes and sleeves may thus help to accelerate toxin transfer from pelagic to benthic compartments, and to spread diarrheic toxins over a more complex trophic web.

Acknowledgments

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CAPÍTULO 4 - Observações preliminares da distribuição em pequena escala de *D.* cf. *acuminata* e *M. rubrum*, sob estratificação térmica, durante um experimento de incubação.

Preliminary observations on small-scale vertical distribution of D. cf. acuminata and M. rubrum under thermal stratification during incubation experiments.

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Resumo

Parte do conhecimento sobre a fisiologia e ecologia do fitoplâncton ocorreu através de experimentos, em campo ou em laboratório. Experimentos com dinoflagelados são realizados majoritariamente com espécies autotróficas. Produtor de toxinas lipofílicas, o cultivo dos mixotróficos *Dinophysis* spp. foi possível somente mediante o provimento de *Mesodinium rubrum* como presa, limitando os experimentos com estes organismos a pequenos volumes. Neste estudo, um foto-biorreator foi montado em uma coluna vertical, com o intuito de se reproduzir uma estratificação térmica ao longo da coluna d´água de um metro de profundidade, observando a distribuição vertical dos organismos planctônicos ao longo do tempo. Amostras naturais contendo *M. rubrum* e *Dinophysis* spp., foram incubadas, por curtos períodos (20–44 h), e observadas as suas interações tróficas em respostas às condições abióticas manipuladas em laboratório. Nós demonstramos a viabilidade técnica e operacional em se reproduzir com precisão, e de forma intermitente, uma estratificação térmica com diferença superior a 3°C. As diatomáceas sedimentaram rapidamente demonstrando que somente a estratificação térmica da água não consegue impedir a sedimentação destes organismos. Dinoflagelados apresentaram padrões de distribuição vertical distintos *C. furca, C. fusus* e *P. micans* buscaram camadas mais próximas a luz e com temperaturas mais elevadas, enquanto que *P. scutellum* e *D.* cf. *acuminata* ocuparam as camadas intermediárias e mais

próximas ao fundo. Os ciliados aparentemente não evitaram as camadas com água mais quente e mais iluminada, distribuindo-se tanto acima como abaixo da termoclina, enquanto que *D.* cf. *acuminata* foi influenciado tanto pela disponibilidade de presas quanto pelas menores intensidades luminosas e/ou temperaturas mais amenas (≤ 20 °C).

Palavras-Chave: Foto-biorreator; dinoflagelados; diatomáceas; termo clina; migração vertical.

Introdução

Grande parte do atual conhecimento sobre a fisiologia e ecologia do fitoplâncton ocorreu através de experimentos conduzidos, em campo ou em laboratório, manipulando variáveis bióticas e abióticas (Gerringa et al. 2000; Gregor & Marsálek 2004; Howard et al. 2007 Laycock et al. 2012; Islabão & Odebrecht 2015). Experimentos em laboratório permitem um melhor controle das variáveis sob investigação, facilita a logística operacional, entretanto, dependem da resistência e adaptabilidade da espécie alvo em sobreviver no cativeiro (Banks et al. 2013; Wang et al. 2013) e, em sua maioria, de uma infraestrutura física e instrumental específica.

O cultivo e os experimentos de laboratório com dinoflagelados são realizados majoritariamente com espécies autotróficas devido à menor complexidade operacional, instrumental e conceitual que estas requerem (Lopez-Rodas & Costas 1999; DeYoe et al. 2007; Galimany et al. 2008; Avila et al. 2012). Mesmo assim, praticamente todo conhecimento sobre quais espécies de dinoflagelados, dentre outros organismos fitoplanctônicos, estão relacionados à produção de compostos tóxicos específicos, foi obtido a partir de cultivos celulares massivos estabelecidos em laboratório. Da mesma forma, a quantidade de toxinas produzida por célula pode ser determinada a partir de experimentos sob condições ambientais controladas e diversas (Gordon et al. 2002; Cruz et al. 2006; Paz et al. 2007; Twiner et al. 2007; Vale et al. 2009; Fux et al. 2011; Riobó et al. 2013; Basti et al. 2015).

Produtor de toxinas lipofílicas, dinoflagelados do gênero *Dinophysis* são responsáveis pela grande maioria dos casos de envenenamento diarreico por consumo de moluscos (DSP) que se registra anualmente ao redor do globo desde a década de 1970 (revisto em Reguera et al. 2014). O cultivo deste organismo mixotrófico em laboratório, entretanto, somente foi exitoso quando estes dinoflagelados foram alimentados com o ciliado mixotrófico fotossintetizante *M. rubrum*, que por sua vez era alimentado com criptofíceas autotróficas, sobretudo do gênero *Teleaulax* (Park et al., 2006). Desde então houve um incremento nos experimentos de laboratório realizados com *Dinophysis* spp. e o conhecimento sobre a ecologia e toxinologia do gênero pode avançar ainda mais (Nagai et al. 2011; Kim et al. 2012; Rial et al. 2012). Entretanto, as condições de cultivo dificilmente conseguem se aproximar das complexas interações ecológicas e características abióticas que estes organismos encontram e requerem no ambiente natural (Marcoval et al. 2007; North et al. 2007). Desta forma, experimentos de incubação de amostras de água em laboratório, normalmente durante florações, têm contribuído complementarmente para o avanço no conhecimento sobre as características relacionadas ao metabolismo energético (Smith et al., 2012) e principalmente em relação ao comportamento ecológico destes dinoflagelados (Nishitani et al. 2008; Garcia-Cuetos et al. 2010; Nagai et al. 2011; Mafra et al. 2014, 2015).

Geralmente experimentos de laboratório com o fitoplâncton marinho, sejam cultivos ou incubações de amostras naturais, ocorrem geralmente em pequenos volumes dentro de frascos tipo Erlenmeyer ou pequenos galões, raramente ultrapassando poucos litros de água (Séchet et al., 2007). Ademais, a dificuldade inerente ao cultivo mixotrófico tem limitado experimentos com *Dinophysis* a volumes ainda menores, em placas de cultivo celular (<10 mL) ou pequenos frascos Erlenmeyer (<250 mL) (e.g. (Nagai et al. 2011; Mafra et al. 2016). Outro importante aspecto está relacionado à altura da coluna d'água utilizada neste tipo de experimentos, que é extremamente reduzida, sendo difícil observar determinados aspectos comportamentais em espécies de interesse, como a migração vertical em *Dinophysis* spp., por exemplo (Reguera et al., 2012). Neste estudo, um foto-biorreator montado em uma coluna vertical foi desenvolvido, com o intuito de se reproduzir e manipular a estrutura físico-química da água ao longo de uma coluna d´água de um metro de profundidade,

observando a distribuição vertical dos organismos planctônicos ao longo do tempo. O reator foi então utilizado para incubar por curtos períodos (20–44 h) amostras naturais contendo *M. rubrum* e *Dinophysis* spp., observando suas interações tróficas e respostas às condições abióticas manipuladas em laboratório.

Materiais e Métodos

Foto-biorreator

O equipamento desenvolvido consiste em um dispositivo mecânico-eletrônico (Fig. 1), construído em PVC (150 mm de diâmetro), envelopado com papel alumínio para evitar a entrada lateral de luz e refração do calor externo incidente. Com 120 cm de altura, o sistema possui uma coluna d'água com exatos 100 cm de profundidade e um volume total de ~18 L. Aberturas de 5 mm de diâmetro, a cada 10 cm, da superfície até 90 cm de profundidade (pré-fundo), permitem obter água de cada estrato amostral sem que haja perturbação das camadas superior e inferior. Acoplado à parede interna do dispositivo, sensores de temperatura (TermoPar®) realizam a leitura da temperatura da água a cada intervalo de tempo determinado (5-120 s). Um aquecedor de água termo estatizado com potência de 200 W, disposto entre 10 e 20 cm de profundidade foi utilizado para gerar a estratificação térmica. Um mini compressor de ar (Boyu®, 30 W), borbulha ar a partir do fundo do dispositivo, gerando turbulência e promovendo a circulação da água dentro do sistema. Uma fonte de iluminação, tipo LED (60 W, luz branca, 6.500K) foi acoplada na porção superior, 20 cm acima da superfície da água. O gerenciamento do sistema e a aquisição das informações de cada sensor de temperatura foi realizado por uma placa de circuito impresso, micro processada (Arduíno®, modelo Mega 250), controlada através de um computador portátil.

Testes preliminares

Foram mensurados os valores de irradiância e a eficiência da aeração na ruptura da estratificação e consequentemente na circulação da água dentro do

sistema (Fig, 2A). Também foi avaliada a capacidade do sistema em gerar e manter uma estratificação térmica por um período de tempo de 30 minutos com medições realizadas a cada 5 minutos, realizados com água do mar filtrada livre de material particulado em suspensão. Foi então avaliada a variação de cada sensor ao longo do tempo durante 5 dias consecutivos e após ajustes no algoritmo que gerencia a aquisição dos sensores conseguiu-se operar com um range de no mínimo 4 °C. Também foi realizado um teste comparativo com um termômetro calibrado e certificado pelo INMETRO, para aferição da calibragem dos sensores do sistema de incubação.

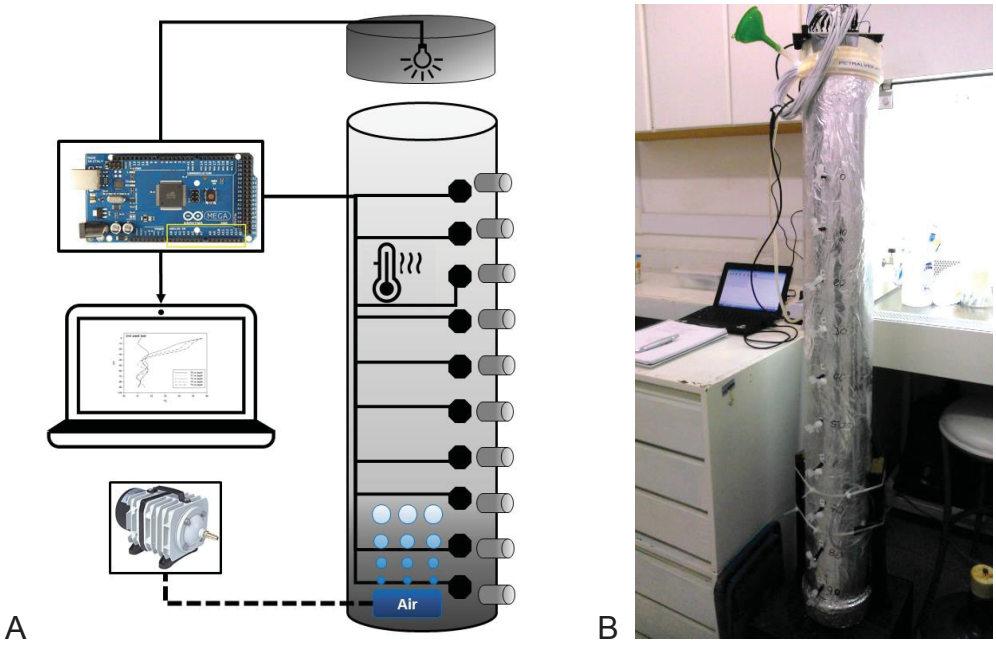

Figura 17 - Cap 4: Fig. 1 – Foto-biorreator desenvolvido: (A) illustração conceitual e (B) fotografia do protótipo utilizado nos experimentos em operação nas dependências do Laboratório de Microalgas (LaMic/CEM-UFPR).

Obtenção do material para incubações

O local escolhido para a coleta das amostras foi a Baía da Babitonga, SC, onde estudos pretéritos indicavam a ocorrência frequente de *Dinophysis* spp. (Mafra et al. 2014; Parizzi et al. 2016; Capítulo 1 da presente tese). Para melhor observar a distribuição vertical dos organismos durante os experimentos de incubação, as amostras iniciais precisavam ter significativa abundancia de organismos, bem como com reduzida quantidade de material particulado em

suspensão (MPS) e predadores (zooplâncton). Assim, 20 litros de água do mar foram coletados com uma rede de plâncton cilíndrico-cônica (50 cm de comprimento por 30 cm de diâmetro e 20 µm de abertura de malha), e passados por uma rede de 60 µm para exclusão do MPS e de predadores.

Delineamento experimental

A abordagem experimental consistiu em incubar a fração selecionada (entre 20 e 60 µm) da água coletada, variando a temperatura e a turbulência associadas a um fotoperíodo de 12:12 h (claro:escuro), para se testar a distribuição vertical do fitoplâncton ao longo do tempo. Durante a incubação, a temperatura da água era constantemente registrada (leituras a cada 2 min) e amostras de água de 50 mL eram periodicamente coletadas a cada 10 cm de profundidade a partir das janelas amostrais (Fig. 1), para quantificação dos organismos planctônicos, incluindo o ciliado *M. rubrum* e o dinoflagelado *D.* cf. *acuminata*, outros dinoflagelados e diatomáceas. Uma fração da água que não foi utilizada no experimento (cerca de 2 L) foi filtrada em filtro de fibra de vidro (0,45 um) e utilizada para repor gradualmente o volume de água removido do sistema durante as coletas, de modo a sempre manter o inalterado o nível de água entre os tempos amostrais. A reposição (500 mL) era feita de forma lenta pela superfície do sistema. Também foi avaliada a turbulência promovida pelo borbulho de ar na ruptura da estratificação e na homogeneização da água dentro da coluna.

Experimento 1

Entre os dias 03.05.2017 e 04.05.2017, foi realizada uma incubação de 20 horas de duração, com 5 intervalos de amostragens (T0-14:00, T1-15:00, T2-19:00, T3-08:00 e T4-10:00 hs). No T0 o sistema estava configurado sem circulação da água, com iluminação e aquecimento da água permanecendo assim por cinco horas (até o T2 quando o aquecimento e a iluminação foram desligados. Durante 13 horas (do T2 até o T3) o sistema permaneceu no escuro sem aquecimento. No T3 foram tomadas amostras ainda na condição sem luz,

que foi acionada imediatamente após a amostragem deste tempo. 60 minutos após o T3 a luz foi novamente desligada, a circulação da água acionada e uma hora depois foi amostrado o T4 encerrando este experimento.

Experimento 2

Nesta segunda bateria, a incubação foi a mais extensa dentre todos os experimentos (44 horas de duração), com 7 intervalos de amostragens (T0-17:00, T1-21:00, T2-09:00, T3-17:00 e T4-21:00, T5- 09:00 e T6-13:00 hs), distribuídos em três dias consecutivos. No T0 o sistema estava configurado com circulação, sem aquecimento da água e com iluminação. Após a amostragem T0, foi acionado o aquecedor, mantido por 4 horas até a amostragem do T1, quando então foram desligados o aquecimento e a iluminação até o final da amostragem do T2 (12 horas após o T1). Durante duas horas após o T0, o sistema esteve sob circulação forçada da água. Do T2 até o T4 (12 horas consecutivas) o sistema permaneceu com aquecimento e com iluminação. Ao final da amostragem do T4 apenas a iluminação fora novamente desligada permanecendo assim pelas próximas 12 horas, até o final da amostragem do T5, quando a circulação da água foi acionada juntamente com o retorno da iluminação. 4 horas após foi amostrado o T6 encerrando este experimento.

Experimento 3

Na última bateria, foi realizada outra incubação desta vez com 36 horas de duração, em três grandes intervalos de amostragens (T0-20:00, T1-17:00, T2-09:00, T3-17:00 e T4-21:00, T5- 09:00 e T6-13:00 hs). Neste experimento à partir do T0 foi acionado o aquecedor e a iluminação, sem circulação da água e mantido assim durante todo o experimento.

Análises microscópicas

A identificação e seleção das espécies a se quantificar durante os experimentos foi realizada previamente, observando-se uma alíquota de 3 mL da amostra inicial (T0) previamente a inoculação dentro do sistema. Neste momento, a presença/ausência de possíveis consumidores do fitoplâncton foi notada. A quantificação das espécies-alvo nas amostras foi feita em uma câmara de contagem de Sedgewick-Rafter (LeGresley & McDermott, 2010) sob microscópio ótico (Olympus AXIO) a um aumento de 200×, quantificando-se entre 210 (Limite de detecção: 4 cél/mL) e 420 (Limite de detecção: 2 cél/mL) quadrados.

Tratamento dos dados

A produção de gráficos e as análises estatísticas comparativas em cada experimento foi realizada com auxílio do software SigmaPlot v.11.0. Foram aplicadas análises da variância (One Way ANOVA) seguidas pelo teste de Tukey para cada espécie-alvo durante os respectivos experimentos. Foram comparada as variações de abundância durante cada tempo entre as profundidades e de cada profundidade entre os tempo, para cada experimento.

Resultados

Testes preliminares

Durante o período de testes preliminares, obtivemos inicialmente uma forte estratificação associada com uma grande oscilação das medidas de temperatura pelos sensores (desvio padrão > 1,5 °C). Um ajuste digital, mediada pela inserção de um fator de correção no algoritmo de controle do sistema, permitiu corrigir os desvios de leitura de cada sensor, resultado em uma faixa de trabalho de leitura com um desvio (discrepância) entre as medidas e sensores na ordem de 0,5 °C (Fig 2B). O teste de durabilidade e reprodutibilidade das leituras e do funcionamento adequado de cada sensor ao longo dos cinco dias consecutivos demonstrou a robustez e confiabilidade dos componentes e

permitiu avaliar o efeito da variação de temperatura do ambiente externo (sala do laboratório) na oscilação da temperatura dentro do foto-biorreator.

Com base nestas informações, ajustou-se o controle de temperatura da sala para que houvesse o mínimo de variação durante o experimento, de modo que a oscilação de leitura dentro do foto-biorreator oscilasse dentro da faixa de 0,5 °C por sensor e entre os sensores (Fig 2C). Por fim, foi determinado tempo necessário para gerar uma estratificação térmica com pelo menos 3°C de diferença, bem como por quanto tempo esta condição de estratificação se mantinha (Fig. 2D). O acionamento do compressor de ar, gerando turbulência ascendente na coluna d'água, rompeu a estratificação homogeneizou a agua em menos de 30 minutos, enquanto que a obtenção da estratificação requer o dobro deste tempo no mínimo.

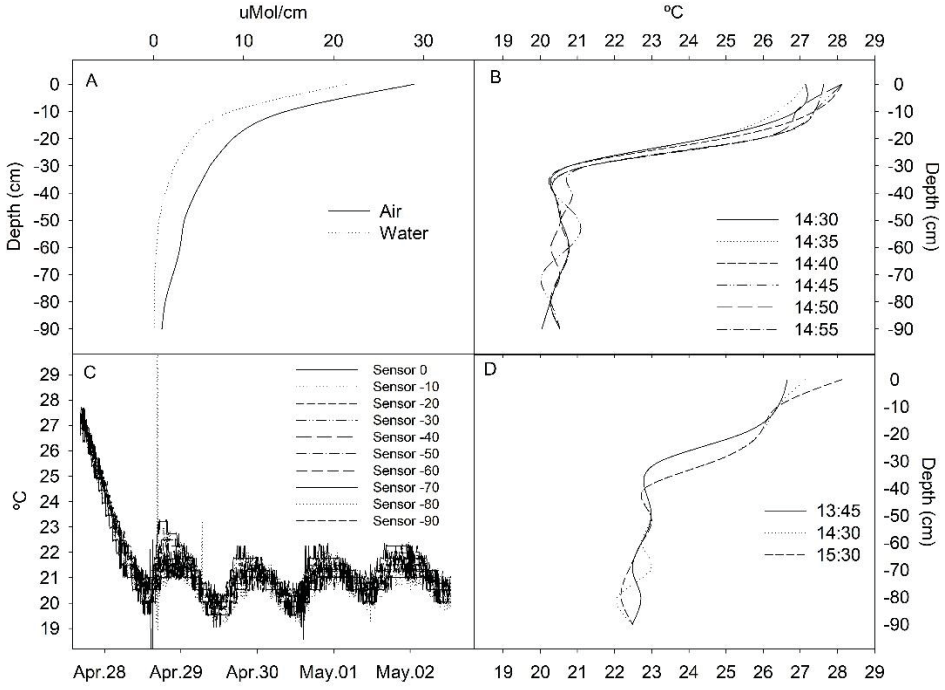

Figura 18 - Cap 4: Fig. 2 – Testes preliminares: (A) Irradiância ao longo do perfil vertical com a coluna vazia (Air) ou cheia de água (Water); e primeiros testes de avaliação da operacionalidade, durabilidade e reprodutibilidade de cada sensor de temperatura durante intervalos de 15 minutos (B) e durante 5 ininterruptos (C); Ajustes fino e confirmação da estratificação durante 3 horas consecutivas (D).

Experimento 1

A amostra inicial inoculada era composta de diatomáceas cêntricas (abundância inicial, T0: 4.761 cél.mL^{-1}) e dos dinoflagelados *Ceratium fusus* (T0: 73.333 cél.mL^{-1}), *Ceratium furca* (T0: 11.428 cél.mL^{-1}), *Prorocenrum micans* (T0:

16.190 cél.mL^{-1}) e *Prorocentrum scutellum* (T0: 5.714 cél.mL^{-1}). No decorrer do experimento, conseguiu-se obter uma estratificação térmica com uma diferença de 4°C entre 10 cm e 40 c*M.* Durante o ciclo noturno, a estratificação foi relaxando gradativamente até que a diferença se reduziu para 2°C entre 0 e 30 cm de profundidade. Após o ciclo escuro, o curto intervalo de tempo até o final do experimento não permitiu reestabelecer a estratificação mais forte (Fig. 3).

Nas primeiras 4 horas de incubação, todos os organismos se distribuíram nos primeiros 40 centímetros de profundidade, principalmente *C. fusus*, a única espécie não observada nas camadas mais profundas. Antes do início do ciclo escuro, todas as diatomáceas ficaram abaixo do limite de detecção (LD: 4 cél/mL) e os dinoflagelados *C. furca* e *P. micans* se concentraram nas camadas mais junto ao fundo. Durante as primeiras 16 horas decorridas, *P. scutellum* foi observado na camada hipotérmica. *C. fusus* permaneceu nas camadas superficiais (hipertérmica) por 7 horas, até meados do período noturno, quando também deixou de ser observado, (Fig. 3), ficando abaixo do limite de detecção.

Experimento 2

A formação de uma termoclina com 4 °C de diferença entre 20 e 40 cm de profundidade ocorreu 4 h a partir do início do experimento. Durante o primeiro ciclo escuro, sem o aquecimento, a estratificação se acentuou e ficou mais acentuada entre 20 e 30 cm de profundidade. Nos ciclos seguintes de fotoperíodo claro:escuro:claro, o aquecimento da água permaneceu operando de forma constante, o que refletiu na elevação da temperatura das camadas intermediárias (de 40 a 70 cm) em 1-2°C, fazendo com que o perfil da estratificação térmica expressasse uma forma de rampa entre a superfície e o fundo.

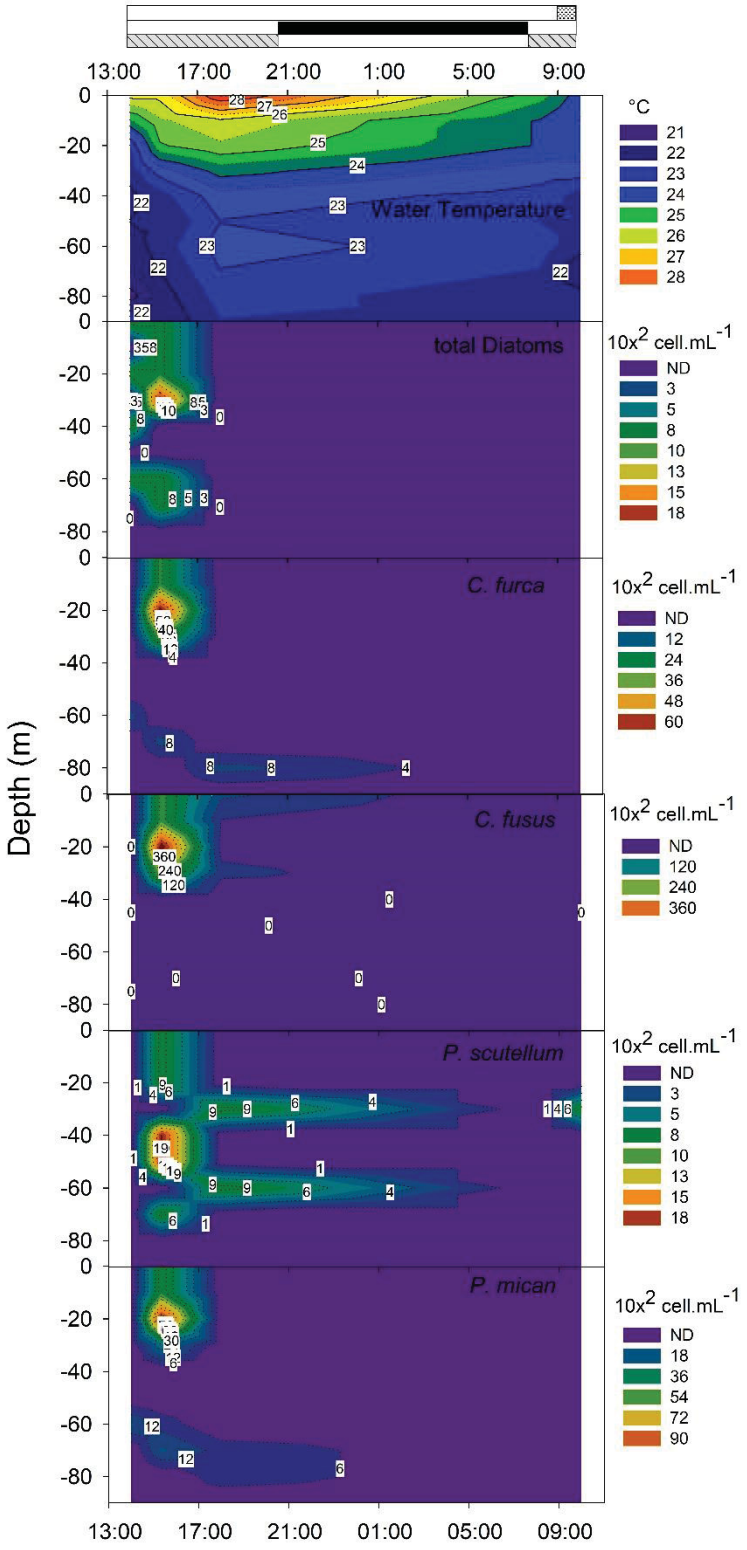

Figura 19 - Cap 4: Figura 03 – Interpolação dos valores de temperatura da água (°C), bem como da abundância (cél.mL^{-1}) de diatomáceas (total) e dos dinoflagelados Ceratium furca, C. fusus, Prorocentrum scutellum e P. micans durante as 20 horas do primeiro experimento de incubação. As barras tracejadas na parte superior da figura indicam quando o compresso de ar estava ligado, as barras pretas e brancas o ciclo de claro:escuro do fotoperíodo e a barra cinza hachurada, o período em que o aquecedor estava ativado na superfície. ND: não detectado.

A amostra inicial de água era composta por duas diatomáceas dominantes, *Skeletonema costatum* (T0: 4.476 cél.mL^{-1}) e *Thalassionema nitzschioides* (T0: 1.319 cél.mL^{-1}), além do ciliado *Mesodinium rubrum* (T0: 123 cél.mL^{-1}) e do dinoflagelado *Dinophysis* cf. *acuminata* (T0: 476 cél.mL^{-1}). Na fase inicial (primeiras 4 horas) deste experimento, a distribuição vertical dos organismos foi uniforme para as diatomáceas, mas as células de *D.* cf. *acuminata* e *M. rubrum* se concentraram nas camadas superiores (0-50 cm), mesmo com a coluna d'água sob circulação forçada durante as primeiras 2 horas (Fig. 3). Após atingir a estabilidade e a estratificação térmica (primeiras 14 horas), a distribuição vertical dos organismos se manteve inalterada com uma leve tendência de sedimentação para as diatomáceas, sendo mais acentuada para *S. costatuM.* Decorridas as 14 horas (no T2), as diatomáceas distribuíam-se nas camadas abaixo da termoclina, tendo *T. nitzschioides* aparentemente sedimentado de forma mais lenta do que *S. costatuM.*

As células de *D.* cf. *acuminata* mantiveram-se acima de 60 cm de profundidade e de *M. rubrum* sobre a termoclina (acima de 40 cm) até o final do segundo período escuro. 14 horas após *D.* cf. *acuminata* apresentou uma agregação na camada de 70 cm de profundidade, associado à porça de água com temperatura mais amena logo abaixo da termoclina, enquanto que sua presa *M. rubrum* ocupava as camadas superiores (Fig. 3). Após 8 horas, células de *D.* cf. *acuminata* foram observadas em menor abundância e restritas às camadas superiores, sobrepondo-se à distribuição dos ciliados. Ao final do segundo período de fotoperíodo escuro (após 36 horas o início do experimento e 12 horas após a última amostragem), todos os organismos encontravam-se abaixo do limite de detecção (LD: 4 cél/L). Com o acionamento da circulação, antes do encerramento do experimento, as diatomáceas e o ciliado *M. rubrum* voltaram a ter sua distribuição junto às camadas superiores,

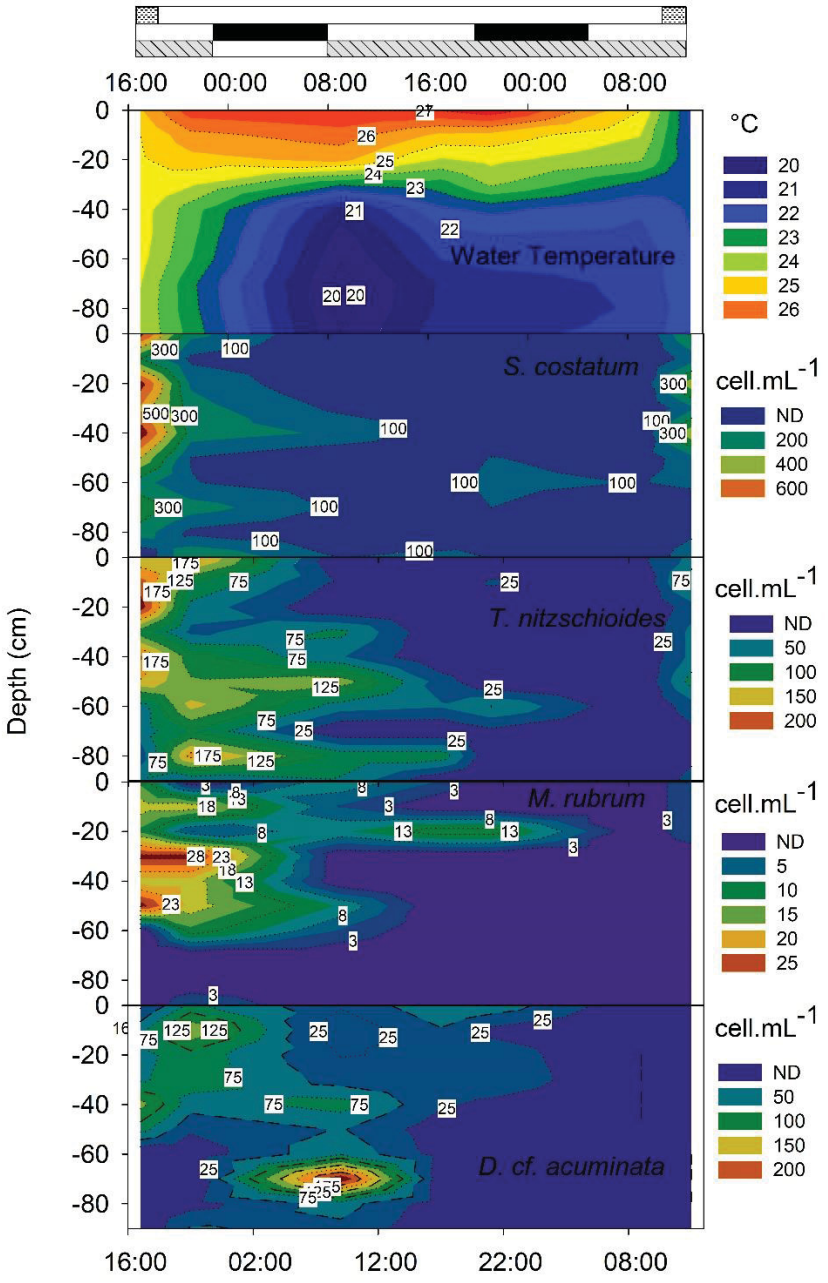

Figura 20 - Cap 4: Figura 04 – Interpolação dos valores de temperatura da água (°C), bem como da abundância (cél.mL^{-1}) das diatomáceas Skeletonema costatum e Thalassionema nitzschioides, do ciliado Mesodinium rubrum e do dinoflagelado Dinophysis cf. acuminata durante as 44 horas do segundo experimento de incubação. As barras tracejadas na parte superior da figura indicam quando o compresso de ar estava ligado, as barras pretas e brancas o ciclo de claro:escuro do fotoperíodo e a barra cinza hachurada, o período em que o aquecedor estava ativado na superfície. ND: não detectado.

Experimento 3

Neste experimento ocorreu a maior diferença de temperatura entre as camadas superior (20 cm) e inferior (40 cm) da água (10 °C). A amostra inicial

era composta por diatomáceas (T0: 2342 cél.mL^{-1}), sobretudo da espécie *T. nitzschioides* (T0: 359 cél.mL^{-1}), além de *M. rubrum* (T0: 28 cél.mL^{-1}) e *D.* cf. *acuminata* (T0: 38 cél.mL^{-1}). Desta vez, a forte termoclina não impediu o padrão constante de sedimentação das diatomáceas, independentemente do fotoperíodo, entretanto, *T. nitzschioides* esteve mais concentrada na profundidade de 40 cm até o final do segundo ciclo claro:escuro (~30 horas após T0), quando voltou a ser observada na camada de 10 cm, com um leve incremento os 60 min de turbulência antes da amostragem final. A abundância de *D.* cf. *acuminata*, sempre maior na camada inferior da coluna d´água, decresceu rapidamente a partir do T0 e deixou de ser detectável (LD: 2 cél/mL) na metade do experimento. O ciliado *M. rubrum* distribuiu-se acima e abaixo da termoclina desde o início do experimento, tendendo a um comportamento agregativo nas profundidades de 10 e 50 cm, além de uma concentração elevada junto ao fundo também, enquanto a termoclina se encontrava a 30 c*M.* Após as primeiras 18 horas, na metade do experimento, a distribuição vertical de *M. rubrum* foi ainda mais restrita à camada superior junto aos 10 cm e mais dispersa nas camadas abaixo da termoclina, com um estreitamento gradativo da distribuição na camada inferior em direção aos 70 cm, permanecendo assim até o final do experimento.

Discussão

Experimentos com organismos marinhos são amplamente difundidos na ciência contemporânea e permitem visualizar e categorizar fenômenos, processos e comportamentos (Goldman 1977; Grzebyk et al. 1994; Ruohonen 1998). A evolução tecnológica das últimas décadas também elevou a capacidade científica de quantificar e de simular em laboratório algumas condições observadas no ambiente (Polo et al. 2014; Louzao et al. 2015; Ojamäe et al. 2016). No presente estudo, demonstramos a viabilidade técnica e operacional em se reproduzir com precisão, e de forma intermitente, uma estratificação térmica com diferença superior a 3°C ao longo de uma coluna de água com um metro de profundidade, o que nos permitiu observar importantes aspectos na distribuição vertical do fitoplâncton marinho e sua influência sobre

as relações tróficas entre o dinoflagelado tóxico *D.* cf. *acuminata* e sua presa, o ciliado fotossintetizante *M. rubruM.*

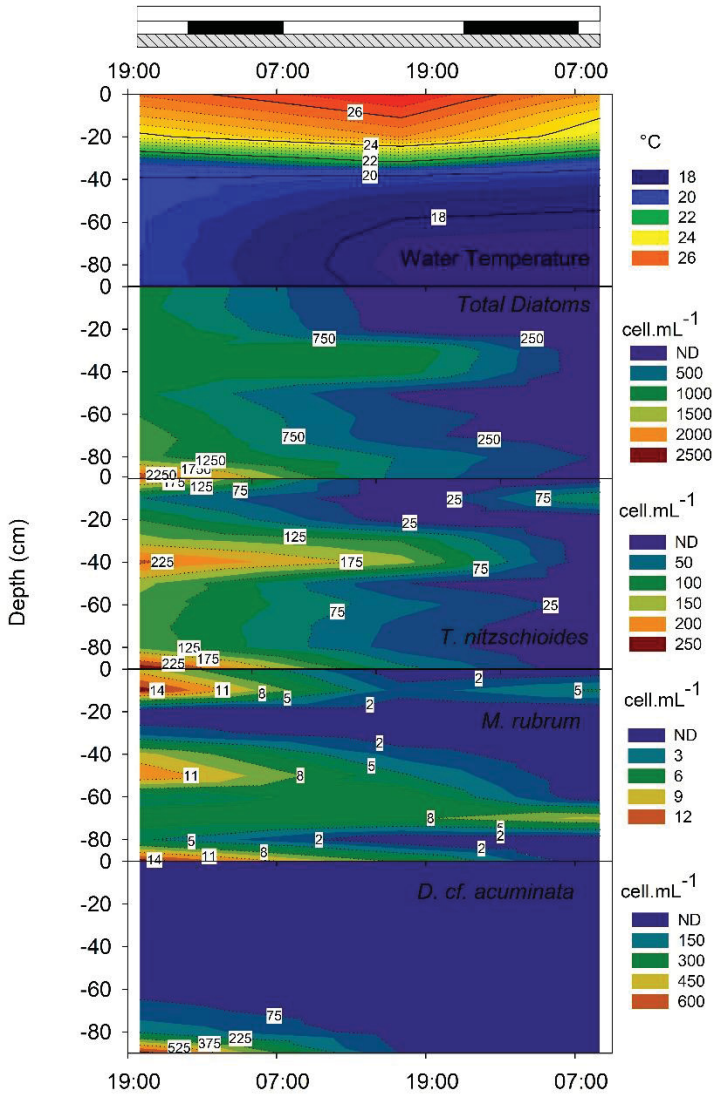

Figura 21 - Cap 4:Figura 05 – Interpolação dos valores de temperatura da água (°C), bem como da abundância (cél.mL^{-1}) das diatomáceas (total), da diatomácea Thalassionema nitzschioides, do ciliado Mesodinium rubrum e do dinoflagelado Dinophysis cf. acuminata durante as 36 horas do terceiro experimento de incubação. As barras tracejadas na parte superior da figura indicam quando o compresso de ar estava ligado, as barras pretas e brancas o ciclo de claro:escuro do fotoperíodo e a barra cinza hachurada, o período em que o aquecedor estava ativado na superfície. ND: não detectado.

Embora a atividade de herbivoria por protozooplâncton, especialmente sobre células menores do fitoplâncton, não possa ser descartada em função da fração de tamanho de partículas selecionada para nossos experimentos (20-60 µm), em nenhum deles a presença de organismos zooplanctônicos que pudessem pastar sobre as espécies-alvo foi quantificável. Assim, grande parte

do decréscimo observado na abundância de algumas espécies-alvo ao longo do tempo deve ser atribuído a perdas por sedimentação, já que a janela amostral mais profunda do fotobiorreator se situava 10 cm acima do fundo (i.e. parte das células pode ter se depositado no fundo, tornando-se indetectáveis em períodos sem turbulência). De um modo geral, as diatomáceas sedimentaram rapidamente, demonstrando que somente a estratificação térmica da água não consegue impedir a sedimentação destes organismos que dependem da turbulência (Ruiz et al., 2004), ainda que algumas espécies coloniais como *S. costatum* e *T. nitzschioides* tenham se concentrado por períodos limitados em estratos intermediários da coluna d´água. Em laboratório, *S. costatum* tolera um amplo range de temperatura (11-30 °C) e demonstra uma forte dependência dos nutrientes inorgânicos dissolvidos como amônio, fosfato e silicato (Takabayashi, 2006).

As diferentes espécies de dinoflagelados apresentaram padrões de distribuição vertical distintos no presente estudo. Enquanto que *C. furca, C. fusus* e *P. micans* aparentemente buscaram camadas mais próximas a luz e com temperaturas mais elevadas (Experimento 1), enquanto que *P. scutellum* e, sobretudo, *D.* cf. *acuminata* ocuparam as camadas intermediárias e mais próximas ao fundo. Similarmente, outros organismos com elevada capacidade natatória, como o dinoflagelado *Gyrodinium instriatum* (Nagasoe et al., 2006) e a rafidofícea *Chattonella ovata* (Yamaguchi et al., 2010), também apresentaram seu melhor desenvolvimento populacional em condições de laboratório com temperatura e irradiância mais elevadas.

D. cf. *acuminata* não demonstrou uma relação íntima com a termoclina, concentrando-se majoritariamente nas camadas mais profundas e frias, exceto durante a transição entre o segundo ciclo claro:escuro no Experimento 2, quando formou um intenso agregado de células logo abaixo da termoclina. Naquela ocasião, entretanto, as células de sua presa (*M. rubrum*) se encontravam concentradas na região da termoclina, ao contrário de outros momentos quando estiveram disponíveis ao longo de quase toda a coluna d'água, indicando que a distribuição vertical de *D.* cf. *acuminata* é fortemente influenciada pela disponibilidade de presas em uma determinada profundidade, conforme sugerido recentemente por Giménez Papiol et al., (2016b) e Mafra et al., (2016a) em

experimentos de pequena escala com *Dinophysis acuta* e *D. acuminata*, respectivamente. Esses estudos mostraram que a captura de presas por *Dinophysis* spp. é facilitada pela produção e liberação de armadilhas mucilaginosas, provavelmente em adição a algum composto tóxico dissolvido de natureza desconhecida (Mafra et al., 2016a), cuja eficiência como mecanismo de captura somente faria sentido se tanto as células da presa quanto as de *Dinophysis* estivessem agrupadas em camadas finas de água, conforme observado no presente estudo. O decréscimo na abundância de *M. rubrum* ao longo do tempo sugere que os ciliados foram gradativamente capturados e consumidos durante nossos experimentos, embora não tenham sido encontradas células de *D.* cf. *acuminata* com presas aderidas.

O rápido decréscimo na abundância de *D.* cf. *acuminata*, como visto no Experimento 2 e principalmente no Experimento 3, poderia estar associado a altas taxas de mortalidade e/ou à concentração de células vivas junto ao fundo do reator por migração. Entretanto, ainda que a turbulência gerada ao final do segundo experimento tenha sido capaz de homogeneizar a coluna d'água e ressuspender colônias de diatomáceas, nenhuma célula do dinoflagelado tóxico foi detectada ao longo do perfil vertical naquele momento. Desta forma, taxas de mortalidade elevadas devem ter sido a principal razão para o desaparecimento das células de *D.* cf. *acuminata* no presente estudo. De fato, uma taxa de mortalidade de 15% associada a uma taxa de crescimento extremamente baixa foi calculada para um período de apenas 22 horas durante uma floração de *D. acuta*, resultando na diminuição da abundância destes dinoflagelados mesmo na camada onde mais se agregavam, a 5 m de profundidade junto à haloclina (Pizarro et al., 2008). Além da possível pastagem por protozooplâncton, como já comentado, a elevada mortalidade de *D.* cf. *acuminata* aqui registrada pode estar associada às altas temperaturas alcançadas abruptamente nas camadas mais superficiais em nossos experimentos. As maiores abundâncias deste dinoflagelado durante as incubações estiveram sempre associadas às camadas de água mais profundas, com menores valores de irradiância (<0,11 µMol/cm) e temperatura (≤20 ºC), temperatura está mais próxima do que a população estava exposta no ambiente natural no momento da coleta (20 ºC). De fato, *D.* cf. *acuminata* demonstra uma maior afinidade por águsa mais frias, muitas vezes

ocupando camadas mais profundas, como previamente observado (Escalera et al. 2006; Díaz et al. 2011).

A distribuição vertical do ciliado *M. rubrum*, por sua vez, não demonstrou relação com a irradiância nem com a temperatura da água, mas foi fortemente influenciada pela presença estratificação térmica da água. Em ambos os experimentos em que esteve presente, o ciliado se concentrou em finas camadas, tanto acima quanto abaixo da termoclina. Além disso, parte das células de *M. rubrum* sobreviveu até o final das incubações, a despeito da provável predação por *D.* cf*. acuminata*, comprovando a alta resistência e adaptabilidade deste ciliado. Em ambiente natural, estes ciliados conseguem sobreviver por longos períodos de tempo sem presas (Myung et al., 2013), sobretudo quando se encontram agrupados em determinadas camadas da coluna d'água, tanto em ambientes tropicais (Bulit et al., 2008) como em regiões polares (van den Hoff & Bell 2015).

Foram identificadas as principais necessidades de melhorias e aprimoramentos como eliminar o calor gerado pelo sistema de iluminação que acaba afetando a primeira e até a segunda camada de água. Também será adicionada uma janela para coleta camada de água rente ao fundo para a observação das células que sedimentare*M.* Dentro do planejamento de desenvolvimento futuro do sistema está a incorporação de mais sensores como salinidade, irradiância, pH e oxigênio dissolvido. As observações preliminares apresentadas são promissoras e animadoras, sobretudo pela inovação conceitual e experimental associada ao desenvolvimento do foto-biorreator. Apesar das limitações apresentadas os resultados permitem inferir que somente a estratificação térmica não consegue suportar pequenas diatomáceas nas camadas superiores da água. Mais importante, os resultados comprovaram em condições de laboratório, e em pequena escala espacial e temporal, a co-ocorrência na distribuição vertical de *D.* cf. *acuminata* e *M. rubruM.* Os ciliados aparentemente não evitaram as camadas com água mais quente e mais iluminada, mantendo sua distribuição tanto acima como abaixo da termoclina sub-superficial, enquanto que *D.* cf. *acuminata* foi influenciado tanto pela disponibilidade de presas quanto pelas menores intensidades luminosas e/ou temperaturas mais amenas (≤20 °C).

Agradecimentos

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CONSIDERAÇÕES FINAIS

A ocorrência do gênero *Dinophysis* (Proença, 1998) e do ciliado *M. rubrum* (Proença, 2004) no litoral sul do Brasil, vem sendo notificada há pelo menos duas décadas. Os resultados obtidos, nas diferentes escalas de observação ao longo deste período de investigação, contribuem para o progresso científico e tecnológico do país, têm relevante significado para o conhecimento ecológico do gênero *Dinophysis* em ambientes costeiros.

A ocorrência de *Dinophysis* do complexo *acuminata* (*D. acuminata* + *D. ovum*), se deu preferencialmente em condições de salinidade entre 26 e 29 PSU, prevalência de nitrogênio (especialmente nitrato) e maior estabilidade da coluna d'água, identificados como os fatores abióticos mais importantes. Além disso, a ocorrência e abundância do ciliado *M. rubrum* demonstrou ser relacionada com a ocorrência do dinoflagelado mixotrófico *Dinophysis* descrito por vários autores (Sjöqvist & Lindholm 2011; Reguera et al. 2012). Durante os *blooms*, foi possível observar o comportamento agregativos destes dinoflagelados em determinada camada de profundidade da água. A salinidade mostrou-se como o fator abiótico mais relevante associado com este comportamento associado aos *Dinophysis*.

O período de maior risco de ocorrência de florações de *Dinophysis* no Litoral Norte de Santa Catarina se estende de junho até outubro, sendo o período mais crítico entre final de julho e o final de agosto, fortemente associados com a contaminação de moluscos bivalves nas semanas subsequentes. Sob condições de *La Niña,* a ocorrência dos eventos de grande abundância dos *Dinophysis* foram mais frequentes e podem estar refletindo as consequências dos processos climáticos que este fenômeno altera no Sul do Brasil. Em diferentes locais do planeta as florações de *Dinophysis* apresentam variabilidades inter-anuais distintas (Reguera et al. 2012; Wells et al. 2015), normalmente relacionadas com fenômeno climáticos (Escalera et al., 2006) e processos oceanográficos de grande escala (Naustvoll et al. 2012; Díaz et al. 2013; Moita et al. 2016). Os resultados apresentados indicam que do final do outono até a primavera, condições meteorológicas de estiagem com redução da velocidade do vento (chuvas e ventos), associada com a influência de massas d'água com baixa temperatura e baixa salinidade, favorecem ao aparecimento e promovem o

rápido crescimento de *Dinophysis*. Acredito que a contra-corrente costeira sob influência da pluma do Rio da Prata (Garcia & Garcia 2008; Möller et al. 2008; Piola et al. 2008) possa ser um dos principais fatores condicionador das florações de *Dinophysis* no sul do Brasil.

Única toxina lipofílica associada com a ocorrência de *Dinophysis* durante o período investigado, o ácido ocadáico (AO) foi detectado, tanto na forma livre, como na forma metabolizada (conjugada ou esterificada), sendo a principal toxina lipofílica, causadoras de DSP (Reguera et al. 2014; Méndez et al. 2016), e condizente com o perfil de toxina do complexo *Dinophysis acuminata* (Raho et al. 2008; Méndez et al. 2016). AO foi detectado desde a fração particulada da água até acumulado em diversos componentes da biota marinha, em alguns casos em quantidades muito superiores aos limites regulatórios estabelecidos, principalmente nos organismos filtradores como os moluscos bivalves. Organismos suspensivos, incluindo duas espécies distintas de bivalves (mexilhão e berbigão) e uma de crustáceos (craca) demonstraram incorporar elevadas quantidades desta toxina diarreica quando expostos ao *bloom* de *Dinophysis*. Gastropodes (principal consumidor dos bivalves), também mostraram acumular expressivas quantidades do AO na forma conjugada, possivelmente já incorporada nesta forma. Pela primeira vez, foi identificada e quantificada a presença do AO em poliquetas, anfipodes e camarões, além da detecção em outros organismos detritívoros e onívoros como caranguejos e peixes respectivamente, todos potencial vetores de transferência trófica destas substâncias. Embora alguns desses organismos estejam associados aos primeiros níveis trófico dentro do sistema marinho, muito são recursos pesqueiros amplamente explorados pela pesca local e podem representar diferentes via de transferência trófica dos contaminantes incorporados, podendo afetar inclusive a saúde humana (Branco 2005; Marenzi & Branco 2005).

Os testes clínicos de rotina não prescrevem claramente o diagnóstico para intoxicações relacionadas à floração de algas nocivas, adicionado ao fato de não haver antídoto conhecido para exposição a estas toxinas, faz com essas doenças afetem negativamente as indústrias locais de pesca e num contexto mais amplo o turismo (Grattan et al., 2016). Neste sentido, os casos de intoxicações alimentares durante eventos de floração merecem ser investigados, pois a assimilação da toxinas pode se dar por diferentes pescados, uma vez que

em uma atualmente se discute rever valor do nível mínimo para o aparecimento de efeitos em humanos (LOAEL – *lowest observed adverse effect level*), em média, 50 µg AO equivalente por pessoa, o que leva a crer que em muitos momentos passados possam ter ocorrido casos leves de intoxicação de consumidores na região de estudo. A ingestão crônica do AO pode causar, além dos efeitos gastrointestinais, alterações de ordem molecular, celular, de expressão genética que podem promover o surgimento de tumores e até o desenvolvimento de câncer (Aune et al., 2007; Ito et al., 2008; Aune et al., 2012; Sosa et al., 2013; Valdiglesias et al., 2013).

Experimentos com organismos marinhos são amplamente difundidos na ciência contemporânea e permitem visualizar e categorizar fenômenos, processos e comportamentos (Goldman 1977; Grzebyk et al. 1994; Ruohonen 1998). O desenvolvimento do foto-biorreator com uma coluna de água de um metro de demonstrou ser tecnicamente viável em reproduzir uma estratificação térmica com diferença superior a 3°C ao longo de uma coluna de água. Isto permitiu observar importantes aspectos na distribuição vertical do fitoplâncton e a influência deste fenômeno físico sobre as relações tróficas entre o dinoflagelado tóxico *D.* cf. *acuminata* e sua presa, o ciliado fotossintetizante *M. rubruM.* As observações preliminares apresentadas são promissoras e animadoras, sobretudo pela inovação conceitual e experimental associada ao desenvolvimento do foto-biorreator. Da mesma forma que o ConCel (ANEXO II), o dispositivo desenvolvido nesta tese (Capítulo 4), continua sendo aprimorado. Acredito que com a incorporação de mais sensores (salinidade, OD, pH), e a possibilidade de controlar a intensidade e o comprimento de onda da luz, importantes lacunas do conhecimento na ecologia do fitoplâncton, especialmente os nocivos, poderão ser elucidadas.

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ANEXO I

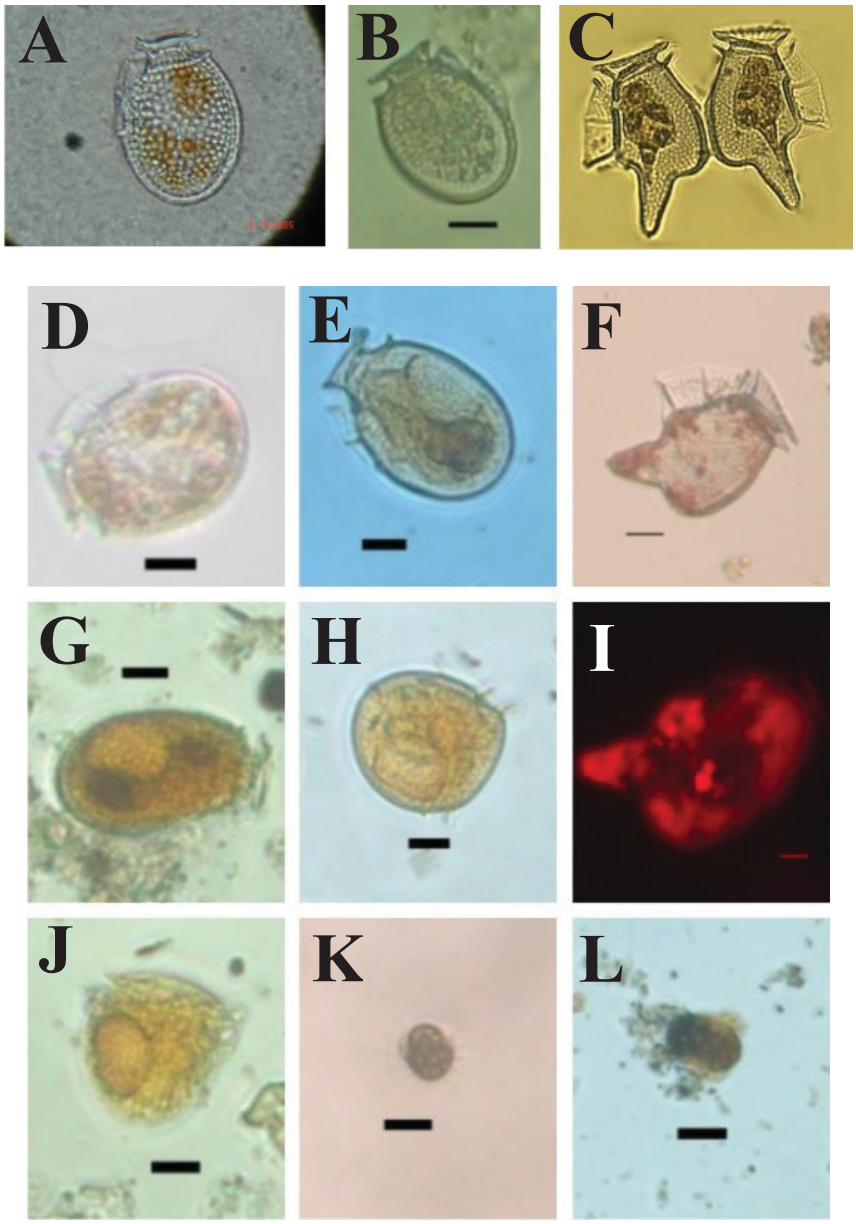

Figura 22 - ANEXO I: Micrografias sob microscópio ótico de Dinophysis cf. acuminata, registrado em 2005 (A, fonte: Proença), registrado em 2006 (B) e registrado durante este estudo (D, E). D. caudata registrado em 2007 (C, fonte: Proença), registrado neste estudo (F) e sob iluminação de epi-fluorescência (I). D. sacculus (G), Phalacroma rotundatum (H), D. scrobiculata (J). Mesodinium rubrum em amostra sem fixador (K) e em amostra fixada com Lugol 1% (L). A barra preta em cada figura indica a distância de 10 µm.

ANEXO II

ConCel: software livre para apoio operacional em microscopia analítica.
ConCel: freeware software for support operational microscope analysis.

Introdução

O software ConCel (Figura 1) foi desenvolvido para aplicação em análises do fitoplâncton, mas que também permite ser utilizado em diferentes áreas do conhecimento que necessitem identificar e quantificar diferentes variáveis e objetos (Houcine et al., 2014; Liu et al., 2017; Ralph, 2018). Com uma interface simples e amigável o ConCel permite ao usuário customizar os parâmetros e variáveis de acordo com suas necessidades analíticas. A interface gráfica foi elaborada para permitir uma rápida identificação das informações apresentadas, bem como facilitar o processo de configurar e preencher os campos de informação. O objetivo da criação deste software consistiu em dar mais celeridade e segurança às análises quantitativas do microplâncton por microscopia ótica, uma vez que ele armazena, calcula e exporta as informações analíticas, e permite realizar diferentes estimativas de quantificação dependendo do método, volume e aumento utilizado. Este software de licença livre (Munir et al., 2018) encontra-se em fase de registro junto ao Instituto Nacional de Propriedade Intelectual (INPI). A versão atual pode ser obtida no endereço eletrônico www.ecounter.com.br

Figura 23 - ANEXO II: Figura 1: Tela principal do software ConCel.

Descrição e funcionalidades

A janela principal está organizada com três opções de menu (Principal, Configurações e Ajuda), contendo campos selecionáveis pré-alimentados, contadores enumerados, e opções selecionáveis de formas de exportação das informações em diferentes formatos gerando um relatório em arquivo texto (*.pdf) e um arquivo de dados tabuláveis (*.csv). No menu arquivo, encontram-se as opções referentes ao armazenamento das informações da análise corrente (“salvar”), o resgate de uma análise armazenada (“abrir”) e o início de uma nova análise (“novo”). O menu configurações permite configurar as informações gerais (“geral”) dos equipamentos analíticos (marca e modelo do equipamento utilizados e grau de magnificação das lentes), configurar a tecla que aciona o contador, cadastrar as variáveis ou parâmetros intrínsecos da análise quantitativa, como as variáveis e o volume da amostra, e cadastrar o operador ou analista. No menu “ajuda”, são apresentadas as informações dos desenvolvedores do software, bem como o manual do usuário em arquivo PDF.

Além de gravar os registros de análises, o ConCel, realiza três formas de tratamento das informações quantificadas (cálculos), que relacionam o volume de amostra analisada, o aumento utilizado na quantificação corrigido pela área total analisada. Adaptável para diversos métodos, o aplicativo possui algoritmos que permitem realizar um tratamento lógico nas informações quantificadas (valor obtido no contador) em relação à área e/ou ao volume informados, exportando o resultado em valores de abundância (densidade celular no caso de microalgas). O desenvolvimento deste software teve como objetivo otimizar o processo de análises laboratoriais de modo a tornar mais ágil e eficiente o processo de análises microscópicas quantitativas, independentemente da área do conhecimento à que se aplica a técnica.

Resultados e Discussão

O uso de instrumentação analítica nas análises laboratoriais é frequente e quase indispensável na ciência moderna (Igathinathane et al., 2006; Houcine et al., 2014; Dorj et al., 2017). Em praticamente todas as áreas do conhecimento, percebe-se a dependência da instrumentação para se poder analisar, estudar, observar processos e fenômenos, que muitas vezes extrapolam os limites sensoriais humanos, principalmente em relação às pequenas escalas

(Maldonado and Barbosa, 2016; Navarro et al., 2016; Pintor et al., 2016; Hernández-Ontiveros et al., 2018). As técnicas analíticas que utilizam equipamentos de microscopia, são amplamente difundidas e praticadas, e em sua grande maioria estão associadas a procedimentos que muitas vezes ocorrem manualmente em meios físicos (papel) que geram resíduos, possibilitam erros variados e requerem espaço de mobília para o armazenamento (Flater, 2018).

Todas as contagens microscópicas realizadas no âmbito desta tese empregaram o uso do software como forma de agilizar a operação e a obtenção de dados. O ConCel permitiu realizar o cálculo analítico resultante da contagem de amostras de fitoplâncton usando tanto a técnica de Utermöhl quanto as câmaras de Sedgewick-Rafter. Também permitiu outras aplicações em análises microscópicas como contagem de alevinos de peixes e larvas de crustáceos, nas atividades pedagógicas do Campus Itajaí, do Instituto Federal de Santa Catarina (IFSC).

A versão não registrada (beta) está em fase de avaliação desde agosto de 2014, sendo diariamente utilizada nas análises de microscopia ótica dentro da rotina operacional do Laboratório de Pesquisa e Monitoramento de Algas Nocivas e Ficotoxinas (LANF). Neste período de desenvolvimento foram identificadas algumas necessidades de ajustes na funcionalidade, operacionalidade e nas exigências técnicas de configuração do software, bem como no processo de aquisição, gerenciamento, armazenamento e tratamento das informações. Foi possível, logo de início observar de forma expressiva a redução em 50% do uso de papel no processo de análises e registro analítico, diminuindo substancialmente a geração de resíduos e a demanda por espaço físico por este procedimento de rotina. Por ser um software livre (*freeware*), espera-se que sua utilização em atividades acadêmicas e científicas seja expressiva, para que se possa atender ao objetivo maior desta iniciativa, ou seja, o desenvolvimento uma ferramenta de auxílio às análises laboratoriais com tecnologia nacional de forma livre e com qualidade, expressando a missão das instituições envolvidas (IFSC e UFPR) em contribuir para o desenvolvimento científico e tecnológico do país.